REMARKS

Reexamination and further and favorable reconsideration of the subject application in light of the following remarks, pursuant to and consistent with 37 C.F.R. § 1.112, are respectfully requested.

The Office Action Summary correctly indicates that claims 148-158, 160, 161, 163 and 164 are pending in the application and stand rejected.

Rejections under 35 U.S.C. § 112

Written Description

Claims 155-157, 160, 161, 163 and 164 have been rejected under 35 U.S.C. § 112 for allegedly failing to comply with the written description requirement. Applicants maintain that the rejection has been traversed for the reasons previously given, but offer the following further reasons in response to the alleged reasons for maintaining the rejection stated in the present Office Action.

The Office has asserted that the methods of claims 155-156 and 163 and the products of claims 157, 160, 161, and 164 rely upon the cells isolated according to the method of claim 148. The Office has alleged that the subject matter of the rejected claims has not been adequately described, because the specification allegedly cannot adequately describe cells which have yet to be isolated. OFFICE ACTION at 2. Thus, the Office implies that no process of manufacture or the product of a process of manufacture can ever be sufficiently described if the process comprises the production of a novel intermediate material that is defined by its method of manufacture. However, the Office has cited no authority that supports such a proposition. Such a proposition would be contrary to well established principals of law and cannot be true.

It is noted that while claim 148 as earlier presented recited "identifying cells," the claim has been rephrased as it currently reads, reciting "isolating cells" to better convey Applicant's intended meaning. That is, the method of claim 148 is a method of manufacture, not of discovery. Cells having the recited properties can be isolated according to the teachings and demonstrations in the specification, as shown by the working examples, and then can be further utilized in the methods of claims 155-157 and 163-64.

Therefore, claim 148 defines a process of manufacture. The cells isolated by the method of claim 148 have distinctive properties of selective induction of a specific immune

response that are recited in claim 148. Claim 157 is a product-by-process claim directed to a composition comprising such cells; and claims 155-156 utilize the product of claim 148. Claims 163 and 164 recite further processing steps that continue from the steps of claim 148.

Product by process claims are proper under 35 U.S.C. § 112. *See, e.g.,* M.P.E.P. § 2173.05(p); *see also, In re Luck*, 476 F.2d 650, 177 USPQ 523 (CCPA 1973); *In re Pilkington*, 411 F.2d 1345, 162 USPQ 145 (CCPA 1969); *In re Steppan*, 394 F.2d 1013, 156 USPQ 143 (CCPA 1967). It is long established that a product may be adequately described as required under 35 U.S.C. § 112, by the method in which it is made. Method claims that recite the use of such products can be no less adequately described than method claims that take one material and make another.

The isolated cell product of claim 148 claimed in claim 157 and utilized in claims 155 and claims dependent thereon, is analogous to a hybridoma producing antibodies. Because the process of making antibodies has been demonstrated to produce cells capable of producing a genus of molecules having defined properties binding to a specific antigen, i.e antibodies. Hybridomas and antibodies claimed by reciting their binding properties, including those yet to be isolated, are held as a matter of law to be adequately described if the antigen used in the method of making the antibodies is fully described. *See, Noelle v. Lederman*, 69 USPQ2d 1508 (Fed. Cir 2004). That is, antibodies are adequately described and claimed by reciting their functional binding properties, which are a result of the process by which they are made. *Id.*

It must be recognized that it is not necessary to sequence or otherwise identify particular molecules that may be presented of the surface of the cells isolated by claim 148 in order to utilize the cells in the methods of claims 155-156 and 163 and describe the claimed products, just as it is unnecessary to define the particular sequence of individual antibodies in order to describe and claim the genus. *Id.* In a manner analogous to the isolation of antibody producing hybridoma cells and the antibodies produced thereby, cells having the properties recited in claim 148 can be isolated and their function confirmed as demonstrated in the working examples of the specification.

Here, the cells isolated by the method of claim 148 are described both in terms of the method of making the cells and by the ability of the cells to induce a specific response from immune effector cells. Therefore, there is no basis for alleging that each of the claimed methods of using the product of claim 148 is not described. Claims 155 and 156 are directed

to a method which takes the product of claim 148 as an intermediate material and produces other isolated cells. Claims 163 and 164 add processing steps to claim 148 that result in a further related product that is, itself, defined by the process used to make it. Claim 161 is directed to a composition comprising the product of claim 155.

Working examples of claims 155-158, 160, 161, 163 and 164, as well as the related claim 148 are provided by Example 4 in the specification. B6 spleen cells were stimulated with TAP-/- and RMA-S.B7-1 cells. TAP was directly ablated by knocking out gene expression in two independent ways. This stimulation of B6 cells reproducibly resulted in cytotoxic responses against RMA-S and TAP -/- targets. The example demonstrates that the response is specific to the novel endogenous self-antigen changes being expressed on the cells that have their TAP function altered. The reproducibility of the stimulation described in the example demonstrates that the claimed methods predictably produce cells having the recited properties.

The Office's alleged basis for the rejection is not supported by fact or law, and the rejection should be withdrawn.

Enablement

Claims 148-157, 160, 161, 163 and 164 have been newly rejected under 35 U.S.C. § 112 for allegedly failing to comply with the enablement requirement. The rejection is respectfully traversed.

The Office has acknowledged that the specification is enabling for the treatment of target cells with an effective dose of a substance that impairs cellular peptide processing for MHC presentation and the isolation of cells which activate CTL that selectively recognize cells showing endogenous epitopes associated with impaired cellular peptide processing for MHC, wherein the cells which are treated are not professional antigen presenting cells. However, the Office has alleged that the specification does not reasonably "provide enablement for the treatment of professional antigen presenting cells with an [effective] dose of a substance that impair cellular processing for MHC presentation and isolation of CTL which selectively recognize cells showing endogenous peptides associated with impaired cellular peptide processing for MHC presentation." OFFICE ACTION at 3 (emphasis added).

Any analysis of whether a particular claim is supported by the disclosure in an application requires a determination of whether that disclosure, when filed, contained

sufficient information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and use the claimed invention. M.P.E.P. § 2164.01. The standard for determining whether the specification meets the enablement requirement was cast in the Supreme Court decision of *Mineral Separation v. Hyde*, 242 U.S. 261, 270 (1916) which postured the question: is the experimentation needed to practice the invention undue or unreasonable? That standard is still the one to be applied. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

The alleged basis of the rejection makes no reference to the factors that must be considered in order to support a contention that a person of ordinary skill in the art would require an undue amount of experimentation to make and use the invention. M.P.E.P. § 2164.01(a). Rather, the Office has merely alleged that one aspect of the invention would not wok as described. In this, the Office is mistaken.

In particular, the Office has alleged as follows:

> The specification states that the cells may be chosen from hematopoietic cells, especially dendritic cells, and that the cells can be healthy cells from an affected tissue or organ (page 10, lines 9-13), which includes cells which are not professional APC. However, the specification then teaches that CTL recognition requires the presence of MHC molecules and the absence of TAP in the target. In the case of choosing dendritic cell to be contacted with the substance that inhibits or eliminates TAP, the dendritic cell would not be able to function as an effector cell for antigen presentation, because the dendritic cell relies on a high level of expression of MHC peptides and co-peptides in the context of T-cell receptor activating ligands. OFFICE ACTION at 3-4.

Applicants respectfully submit that the Examiner has not fully apprehended the teaching of the specification. Dendritic cells (DC) do function as taught by the specification, although they express lower levels of MHC class I than normal DC do. They do this in a novel way. These novel DCs do not express normal epitopes either, only normal TAP-independent epitopes that we are already tolerized to plus most importantly for the first time the new and novel type of antigen Applicants have created selective immune response to by the invention.

To be and function as an antigen presenting cells (APC) the most important and unique aspect is the ability for costimulation. This part the skilled artisan would know. Professional APC such as dendritic cells (DC) differ from all other cells (non-APC) in several unique and important ways. What make them most unique are the B7 co-stimulatory molecules they express on the cell-surface. Mature DC are the most efficient professional

APC and constitute the functional APC cells in lymph-nodes and experimental spleen cell cultures. B7 levels are strongly up-regulated on maturing dendritic cells. B7 is also most relevant to this invention since transfecting non-APC cells such as the RMA-S with high levels of B7 make them into functional APC tools mimicking real DC. See *e.g.*, the following supplied references regarding B7 for a relevant understanding of the knowledge in the scientific literature: Role of Costimulators in T Cell Differentiation, Schweitzer et al., *Journal of Immunology*, 158: 2713-2722, 1997 (Exhibit A). Changes in the strength of co-stimulation through the B7/CD28 pathway alter functional T cell responses to altered peptide ligands, Murtaza et al., International Immunology, 1999, Vol. 11, No. 3, pp. 407–416 (Exhibit B).

The Office has further alleged:

> [T]he dendritic cell would then be reminiscent of the target. The altering of the plurality of MHC peptides displayed by dendritic cell would alter the T cell receptors which would be complementary to the displayed dendritic cell antigens. OFFICE ACTION at 4.

Applicants respectfully submit that this is not a correct understanding of the scientific knowledge at the time of filing or presently. Professional APC can specifically induce, expand and activate T-cell immunity to what they express on their MHC class I molecules. Non-APC can not do this, instead what they express on their MHC class I molecules makes them only targets, or not, for already activated T-cells. For more information the Examiner is directed to: Janeway, Charles A. Jr., et al., IMMUNOBIOLOGY: the immune system in health and disease, (2001, 5th ed. Garland Publishing, New York.) (relevant excerpts presented as Exhibit C).[1]

The Office has not met its burden to support the rejection. The Office's entire alleged basis has been summarized by the Office as follows "There is no evidence in the specification or any art of record to suggest that the limiting the CTL activated by the dendritic cell will provide CTL which recognize the TAP deficient antigen on the target cells." OFFICE ACTION at 4. Upon careful review, it is apparent that the implied lack of operability is not founded in

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[1] Table of Contents at http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=imm.TOC&depth=2
see Chapter 8 at
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Books&cmd=Search&term=dendritic+cells+AND+imm%5Bbook%5D&doptcmdl=TOCView
see Chapter 14 at
http://www.ncbi.nlm.nih.gov/books/bv.fcgi?highlight=rejection,antigens,Tumor&rid=imm.section.2184#2185.

fact. However, even if true, the Office's allegation would be insufficient to make an enablement rejection. *See*, M.P.E.P. § 2164, et seq.

In order to make a rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. M.P.E.P. § 2164.04 (citing *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure)). A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. M.P.E.P. § 2164.04.

The methods of the invention have been demonstrated by working examples in the specification. Applicants respectfully submit that the implications of the Office to the contrary are not supported by evidence and the Office's reasoning has been shown to be misdirected and/or incorrect as applied to the present invention by the evidence presented in the Exhibits attached hereto. Because the Office has not adduced any sound scientific basis supported by evidence for doubting that the invention can be made and used as described, let alone provided an proper analysis explaining why any deficiency would impose a burden of undue experimentation upon a person of ordinary skill, the Office has not met its burden to support this rejection. M.P.E.P. § 2164.04(a). Accordingly, withdrawal of the rejection is appropriate and respectfully requested.

Claim 158 has been newly rejected under 35 U.S.C. § 112 for allegedly failing to comply with the enablement requirement. The rejection is respectfully traversed.

The Office has alleged:

> The specification has not provided evidence that the antigen recognized by the CTL generated against the TAP-deficient variant of RMA is a universal tumor antigen, nor does the specification provide any evidence that a an immunological effector cell which selectively recognized cells showing impaired cellular possessing would be efficacious in vivo.
> OFFICE ACTION at 4.

Applicants respectfully submit that the results shown in the application demonstrate that T-cells stimulated by RMA-S.B7-1 are capable of recognizing several different target cells including human cell lines transfected with mouse MHC molecules. This shows that the antigen expressed is not specific for the tumor cell line RMA-S but is expressed on a wide range of cells as well as human cells is shown in figure 2. That the effector cells would be efficacious in vivo is shown in figure 4

These findings have been confirmed by van Hall et al, *Nature Medicine*. 12(4):417-24, 2006 (attached as Exhibit D). The peer reviewed published results support the teaching that T-cell reactivity observed is specific for cells with deficient antigen presentation rather than for one tumor antigen expressed only on RMA-S as well as the in vivo efficacy.

The Office has further cited Ohlen et al (Journal of Immunology, 2001, Vol. 166, pp. 2863-2870); Antoinia et al (International Immunology, 1995, Vol. 7, pp. 715-725); and, Yu and Restifo (Journal of Clinical Investigation, 2002, Vol. 110, pp. 289-294, especially page 292). OFFICE ACTION at 4-5.

Applicants respectfully disagree with the interpretation of these publications alleged by the Office. The articles by Ohlen et al elegantly shows that even in vivo, anergic T cells reactive with tumor-associated antigens can be recovered by repetitive in vitro stimulation and can mediate efficient tumor therapy when adoptively transferred. That Ohlen et al. suggests such strategies should be further developed does not reflect adversely on the teachings of the present specification. Indeed, Ohlen et al. supports a conclusion that the teachings of the present specification are enabling.

The article by Antoinia et al illustrates the problems of loss of Th1 activity as a consequence of the thymic epithelium being encountered by tissue-specific proteins results in the functional tolerization of CTL in vivo, despite the fact that CTL are fully functional in vitro. In this way autoimmune destruction is contained. Thymic expression of peripheral proteins may therefore be an additional way in which tolerance to peripheral proteins can be achieved. Applicant's invention and the novel immune response generated would not be affected by this tolerizing problem since these novel antigen type would never be presented in the thymus. This is an advantage of the presently claimed invention that one skilled in the art would recognize as a non-obvious feature and improvement over other immunotherapy inventions.

The review by Yu and Restifo brought forward by the Office is an excellent review for understanding exactly why the current invention is so relevant and unique. Lack of efficiency in the other types of immunotherapy inventions that are being reviewed is not relevant grounds for rejecting the presently claimed invention. The presently claimed invention provides a specific way to address and solve fundamental flaws in the other tried immunotherapy inventions.

Indeed, the efficiency of the presently claimed methods has been tested, proven and now published in a peer reviewed publication of the highest scientific standard in Nature Medicine. *See,* van Hall et al, *Nature Medicine, supra.* (Exhibit D). With regard to claim 158 in particular, see Fig. 1g in the paper regarding adoptive transfer of TEIPP-specific T cells. The figure shows that these T cells can exert a marked antitumor effect in vivo, in that one-half of the treated group totally rejected the highly aggressive tumor, plus the other half that did not, showed a clearly delayed tumor outgrowth. *Id.*

The Office has asserted that claim 158 requires, as a prerequisite, carrying out the methods of claims 148 and 155, and that doing so would constitute undue experimentation. Applicants respectfully note that the Office has failed to provide any analysis of the factors required to make such a determination. M.P.E.P. § 2164.01(a). Even if one were to start from the beginning with the method of claim 148, the method requires only an application of the methods described in the specification. It must be noted that as a matter of law, the fact that any required experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm"n 1983), aff'd. sub nom., *Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985). *See also In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404.

The Office has adduced no analysis of the factors that must be considered in order to support a rejection on enablement grounds. The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. *In re Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219 (CCPA 1976). That is why the Manual of Patent Examination Procedure requires an analysis of the relevant factors before an enablement rejection can be made. M.P.E.P. § 2164.01(a) It is improper to conclude that a disclosure is not enabling based on an analysis of only one of the above factors while ignoring one or more of the others. The examiner's analysis must consider all the evidence

related to each of these factors, and any conclusion of nonenablement must be based on the evidence as a whole. *Wands*, 858 F.2d at 737, 740, 8 USPQ2d at 1404, 1407.

Because the Office has failed to adduce any evidence that the claimed invention cannot be practiced as described in the specification without undue experimentation, or even any analysis of the factors that must be considered before making such a determination, the Office has not met its burden. Accordingly, the rejections alleging a lack of enablement should be withdrawn and such action is respectfully requested.

CONCLUSION

In view of the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order. Such action is earnestly solicited.

In the event that there are any questions relating to this Amendment and Reply or the application in general, it would be appreciated if the Examiner would telephone the undersigned concerning such questions so that prosecution of this application may be expedited.

The Director is hereby authorized to charge any appropriate fees that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

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EXHIBIT A

Role of Costimulators in T Cell Differentiation

Studies Using Antigen-Presenting Cells Lacking Expression of CD80 or CD86[1]

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For T cells to be optimally activated, recognition of Ag/MHC complexes by the TCR must be accompanied by a second, costimulatory signal that can be provided efficiently by the related costimulatory molecules CD80 (B7-1) and CD86 (B7-2). Recently, CD80 and CD86 have been implicated as differential determinants of Th1- vs Th2-type cytokine profiles. However, this remains a controversial issue since conflicting results have been obtained in different experimental models both in vivo and in vitro. To investigate the role of CD80 and CD86 in Th subset differentiation, we have examined the cytokine profiles induced in TCR transgenic T cells stimulated by peptide in association with splenic APCs obtained from knockout mice that selectively lack expression of either the CD80 or the CD86 molecule. Our data suggest that CD86, and to a lesser extent CD80, can make significant contributions to the production of both IL-4 and IFN-γ. However, neither molecule plays an obligatory role in priming for the production of either effector cytokine. Furthermore, CD80 and CD86 contribute to the magnitude of T cell activation, but do not appear to selectively regulate Th1 vs Th2 differentiation. *The Journal of Immunology,* **1997, 158: 2713–2722.**

The structurally related costimulatory molecules CD80 and CD86, which are expressed on APCs, deliver a potent signal through the T cell surface molecule CD28 (1–5). Ligation of CD28 provides a crucial costimulatory signal that permits the effective Ag-induced activation of T cells and prevents the induction of anergy (6–15). However, it is now apparent that the CD80/CD86-CD28/cytotoxic T lymphocyte-associated Ag-4 (CTLA-4)[3] pathway may play a more complex role than initially thought. Although CD28 −/− mice show deficient Ab responses in vivo, cellular immunity is to some extent functional (16), and T cell proliferation, albeit reduced, can be induced in vitro in a CD80/CD86-independent manner (1). Indeed, high Ag doses effectively initiate CD28-independent activation and proliferation, although induction of high levels of IL-2 production and sustained proliferation are CD28 dependent (14, 17). CD80 and CD86 also bind CTLA-4, an Ig superfamily member with homology to CD28. Recent studies indicate that CTLA-4 can deliver a negative signal to activated T cells (18–21).

One question that is currently arousing considerable interest is whether CD80 and CD86 are functionally distinct. CD80 and CD86 have only 25% amino acid identity, with marked differences in their cytoplasmic domains (22, 23), and utilize distinct CTLA-4-binding determinants with distinct dissociation kinetics (24). In addition, both the temporal and spatial expression of CD80 and CD86 are differentially regulated. CD86 is expressed constitutively on dendritic cells, macrophages, and T cells, and expression is rapidly induced or enhanced on APCs and T cells in response to numerous stimuli, whereas CD80 appears more slowly and often at significantly lower levels (4, 5, 25–31). Thus, functional differences between CD80 and CD86 could potentially arise due to either inherent structural differences generating distinct biochemical signals or differential ligation of CD28 and CTLA-4 as a result of different expression kinetics.

In many experimental systems, immune responses are blocked in an additive or synergistic manner by mAbs directed against CD80 and CD86 (3–5, 21, 23, 25). Blocking CD86 often has a more potent effect than blocking CD80, presumably reflecting the differential patterns of CD80 and CD86 expression. However, administration of anti-CD80 and anti-CD86 mAbs can result in functionally different outcomes with respect to cytokine production by $CD4^+$ T cells (32, 33). In particular, anti-CD80 and anti-CD86 mAbs can differentially influence the induction of Th1 responses (characterized by production of IL-2, IFN-γ, and TNF-β, and associated with inflammatory cell-mediated immunity) and Th2 responses (characterized by production of IL-4, IL-5, and IL-10, and associated with the down-regulation of inflammation and with certain forms of humoral immunity) (34). Specifically, treatment with mAb directed against CD80 has been shown to ameliorate the Th1-mediated autoimmune disease experimental autoimmune encephalomyelitis (EAE), whereas treatment with mAb directed against CD86 exacerbated disease, at least under suboptimal conditions of disease induction (33). These results are consistent with the ability of CHO transfectants expressing CD86 to induce IL-4 as well as IL-2, whereas CHO transfectants expressing CD80 induced IL-2 only (35). On the other hand, anti-CD86 treatment was effective at depressing Th1-mediated diabetes in nonobese diabetic

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[3] Abbreviations used in this paper: CTLA-4, cytotoxic T lymphocyte-associated antigen-4; CHO, Chinese hamster ovary; EAE, experimental autoimmune encephalomyelitis; PE, phycoerythrin.

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mice, and anti-CD80 treatment exacerbated disease (32), suggesting that the relationship between CD80/CD86 and cytokine production may not be simple.

Other studies, however, have failed to demonstrate a functional dichotomy between CD80 and CD86 (36, 37). Blockade of the CD80/CD86-CD28/CTLA-4 pathway during primary T cell stimulation inhibits production of both IL-2 and IL-4 (38–40), although CD80/CD86-CD28/CTLA-4 pathway blockade at the time of infection with *Leishmania major* unexpectedly skews the usual Th2 response of susceptible mice toward a curative Th1 response (41). Following their induction, differentiated Th1- but not Th2-type responses are sensitive to CD80/CD86-CD28/CTLA-4 blockade in a number of in vitro and in vivo systems (42–46). The latter studies suggest that CD80/CD86-mediated costimulation plays distinct roles at different stages of T cell activation, being critical for inducing Th2 responses during initial priming, and for maintaining Th1 responses.

In this study, we use APCs isolated from mice that lack expression of either CD80 or CD86 to investigate the influence that the specific lack of one or the other molecule on the APC exerts on the cytokine profile of responding TCR transgenic T cells. We have focused on the role of CD80 and CD86 molecules during the initial priming of T cells. These studies avoid the potential complications and artifacts, such as FcR-mediated effects and cross-linking, that might arise when mAbs are administered as blocking agents. Furthermore, this system allows the role of CD80 and CD86 molecules on APCs, rather than T cells that also express these two molecules (25, 47–50), to be addressed in a definitive way. Our results indicate that CD80/CD86-mediated costimulation significantly contributes to both IFN-γ and IL-4 production, especially when T cells are primed under suboptimal conditions, with a greater influence of CD86 than CD80. However, neither CD80 nor CD86 has an obligatory role in priming for production of a particular cytokine when T cells are primed under optimal or supraoptimal conditions. Our studies suggest that the apparent differences in the cytokine profiles observed in various experimental systems using anti-CD80 and anti-CD86 mAbs, and attributed to differential roles for CD80 and CD86, can be accounted for by differences in the efficiency of T cell priming as a result of the distinct timing and level of expression of CD80 and CD86.

Materials and Methods

Animals

Animals were maintained in a pathogen-free facility and used at 8 to 12 wk of age. DO11.10 TCR transgenic mice, which recognize OVA peptide 323–339 (see below) in association with I-A^d (51), were kindly provided by Dr. Dennis Loh (Washington University, St. Louis, MO) and maintained in our facility by breeding with BALB/c mice. BALB/c mice were obtained originally from Taconic Laboratories (Germantown, NY) and bred within the facility. CD80 −/− mice were derived on a 129 background (23) and then bred to at least backcross F9 onto BALB/c. CD86 −/− mice, also derived on a 129 background (F. Borriello, M. Sethna, A. N. Schweitzer, E. Tivol, D. Jacoby, T. B. Strom, G. J. Freeman, and A. H. Sharpe, unpublished experiments), were backcrossed for at least four generations with BALB/c for use in these experiments and screened by FACS analysis to ensure expression of class II I-A^d.

Peptide

OVA peptide 323–339 ($OVA_{323–339}$) was obtained, HPLC purified, from the Beckman Center, Stanford University Medical Center (Palo Alto, CA). The amino acid sequence was as follows: Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-COOH.

Antibodies

mAbs used in the purification of APCs and T cells were produced from hybridomas obtained from American Type Culture Collection (Rockville, MD) (GK1.5 (anti-CD4); ADH4 (anti-CD8); M5/114 (anti-class II I-$A^{b,d}$, I-$E^{d,k}$); and 11B11 (anti-IL-4)) or purchased from Serotec (Oxford, U.K.) (anti-Thy-1.2). Directly conjugated mAbs used for cell surface staining and FACS analysis (anti-CD3 FITC or anti-CD3 PE; rat IgG2a FITC; hamster IgG PE; anti-CD80 FITC (clone 1G10) or CD80 PE (clone 16-10A1), as indicated; anti-CD86 FITC (clone GL1); and anti-I-A^d FITC) were obtained from PharMingen (San Diego, CA).

Cell preparations and cultures

APCs were prepared from whole spleen cells depleted of T cells by treatment with anti-CD4 (GK1.5), anti-CD8 (ADH4), and anti-Thy-1.2 mAbs, followed by rabbit Low-Tox complement (Accurate Chemical & Scientific Corporation, Westbury, NY), and treated with 50 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, MO). T cells were prepared from pooled spleen and lymph node cell suspensions from DO11.10 mice. Adherent cells were removed by passage over nylon wool columns. Remaining APCs and $CD8^+$ T cells were removed by treating recovered cells with anti-CD8 (ADH4) and anti-class II (M5/114) mAbs followed by complement. T cells (2×10^5/ml final concentration, unless otherwise stated) were incubated with mitomycin C-treated APCs (10^6/ml final concentration, unless otherwise stated) with or without $OVA_{323–339}$ in RPMI 1640 supplemented with 10% heat-inactivated FCS (Sigma Chemical Co.), 5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, 10 mM HEPES (all from Life Technologies, Grand Island, NY), and 15 μg/ml gentamicin (BioWhittaker, Walkersville, MD). Cultures were established in 24-well plates at 2 ml/well, or 96-well plates at 200 μl/well. After 4 or 5 days, dead cells were removed by density gradient separation over Ficoll-Hypaque (Organon Teknika, Durham, NC), and the remaining T cells were rested overnight in media before restimulating in 24-well or 96-well plates at 10^6/ml (final concentration) on 10^6/ml (final concentration) fresh APCs obtained from wild-type BALB/c mice, in the presence of 1 μg/ml $OVA_{323–339}$.

Cytokine analysis

Cytokine levels were analyzed by ELISA performed on supernatants collected 48 h after the initiation of either primary or secondary culture. mAbs and recombinant cytokine standards used in the ELISAs were obtained from PharMingen for IL-2 and IL-4, or Genzyme Corp. (Cambridge, MA) for IFN-γ. Lower limits of detection, as determined using a standard curve, were as follows: for IL-2, 150 pg/ml; for IL-4, 40 to 80 pg/ml; and for IFN-γ, 30 to 120 pg/ml.

Proliferation

Proliferation was assessed by addition of 1 μCi/well of [^{3}H]TdR (DuPont NEN, Boston, MA) to wells of a 96-well plate for the terminal 6 h of a 72-h primary culture or for 18 h following 48 h of secondary culture. Incorporated radioactivity was measured by liquid scintillation counting.

Detection of CD80 and CD86 expression by cell surface staining and FACS analysis

Whole spleen cell suspensions or mixed lymph node cell suspensions prepared from DO11.10 TCR transgenic mice were stimulated for 4 days with 10 μg/ml LPS (Sigma Chemical Co.) and 20 μg/ml dextran sulfate to confirm the genotype of wild-type, CD80-, and CD86-deficient mice, or with $OVA_{323–339}$, as indicated in the text. Before staining, dead cells were removed by density-gradient centrifugation on Ficoll-Hypaque (Organon Teknika). Cell suspensions were stained with the indicated fluorochrome-conjugated mAb obtained from PharMingen at a final concentration of 5 μg/ml in PBS containing 1% BSA (Sigma Chemical Co. fraction V) and 0.02% sodium azide. Stained cells were analyzed on a FACStar^{Plus} machine (Becton Dickinson, Mountain View, CA) using CellQuest software for Macintosh.

Results

Determination of conditions favoring concurrent induction of both Th1- and Th2-associated cytokines

To investigate the effect of CD80 or CD86 deficiency upon the induction of Th1 and Th2 cytokines, we first needed to identify conditions under which we could reliably detect concurrent production of significant levels of IL-2, IFN-γ, and IL-4 under CD80/CD86-sufficient conditions. We used a culture system described by Hsieh et al. (52) in which we could measure cytokine production during both initial priming and subsequent restimulation of T cells. This involved primary culture of DO11.10 TCR transgenic T cells,

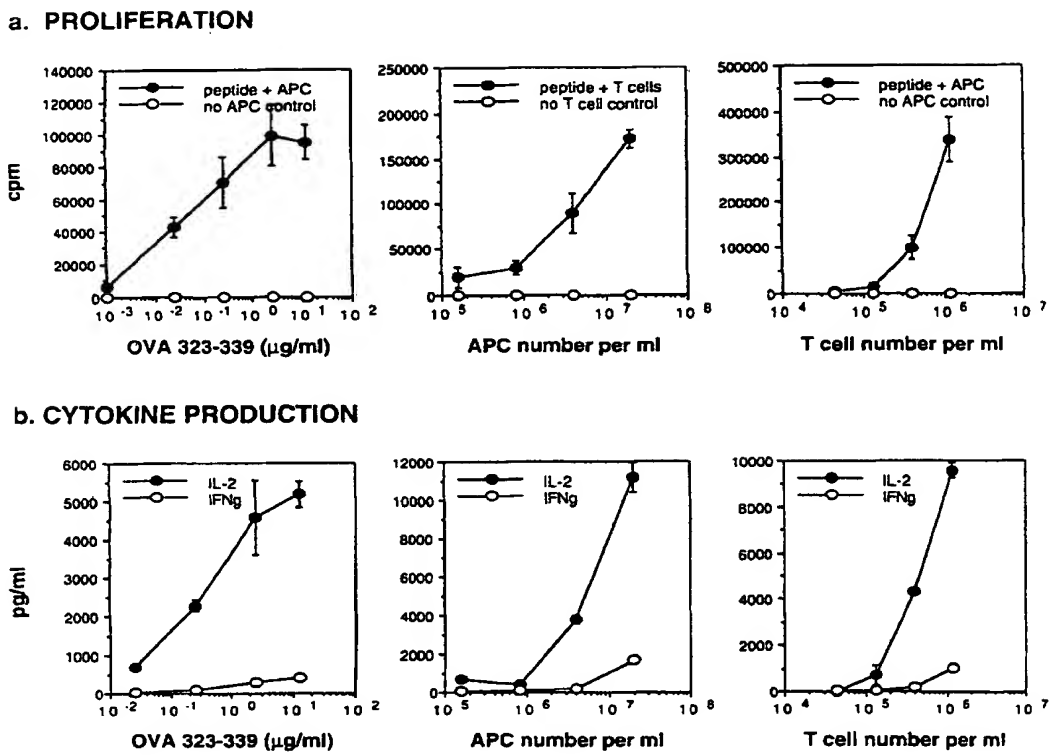

FIGURE 1. Primary proliferative responses and cytokine production of DO11.10 TCR transgenic T cells following stimulation with a range of peptide concentrations, APC numbers, or T cell numbers. Purified $CD4^+$ DO11.10 T cells were stimulated in primary cultures with wild-type T-depleted spleen cells in the presence of $OVA_{323-339}$. The number of APCs, the number of T cells, and the concentration of Ag were titrated in turn, as indicated on the *x*-axis, all other parameters remaining constant (4×10^6/ml APCs, 4×10^5/ml T cells, and 2.5 μg/ml peptide). Supernatants were recovered after 2 days of culture, and the level of cytokine production was determined by ELISA. Proliferation was measured for the final 18 h of a 90-h culture. No IL-4 was detected by ELISA. Cell concentrations mentioned in figure and legend refer to cell suspensions before fourfold dilution upon addition to wells. Error bars indicate SE.

mitomycin C-treated APCs, and $OVA_{323-339}$ peptide for 4 to 5 days (priming), after which viable T cells were recovered by density-gradient centrifugation. After resting overnight in fresh medium, these T cells were restimulated by peptide in the presence of mitomycin C-treated APCs (restimulation).

Upon primary culture of DO11.10 TCR transgenic T cells with wild-type BALB/c APCs, the level of proliferation and IL-2 production increased in a dose-dependent manner with respect to Ag concentration, APC, or T cell number. Only low levels of IFN-γ were detected, showing a similar pattern to IL-2 production (Fig. 1), and IL-4 was undetectable by ELISA. The number of T cells recovered after priming correlated with the relative level of T cell proliferation measured during the primary response (data not shown). For restimulation studies, T cell concentrations were adjusted so that equivalent numbers of T cells were added to each of the secondary cultures. T cell proliferation upon secondary stimulation was relatively constant across the spectrum of primary culture conditions (Fig. 2*a*). In contrast to the primary response, significant levels of IL-4 and IFN-γ were detected following a second round of stimulation. However, the relative levels of the three cytokines varied dramatically, depending on the conditions under which the T cells had been primed. The highest levels of IL-4 were observed consistently at the highest priming doses of Ag, or highest numbers of APCs or T cells. In striking contrast, the level of IL-2 and IFN-γ upon secondary stimulation remained steady or decreased with increasing doses of Ag or cell number in the primary culture (Fig. 2*b*). Thus, the magnitude of the primary T cell response, as reflected in the proliferation and levels of cytokine production, is an important determinant of the extent of IL-4 production independent of the level of IFN-γ production.

Primary responses following stimulation with CD80 −/− and CD86 −/− APCs

To investigate the role of CD80 and CD86 during T cell priming, wild-type APCs were compared during primary culture with APCs obtained from mice genetically lacking expression of CD80 (23) or CD86 (F. Borriello, M. Sethna, A. N. Schweitzer, E. Tivol, D. Jacoby, T. B. Strom, G. J. Freeman, and A. H. Sharpe, unpublished observations). Figure 3 shows the respective lack of CD80 and CD86 expression on spleen cells from these two strains following stimulation with LPS and dextran sulfate.

Upon primary culture with peptide and CD80 −/− or CD86 −/− APCs, DO11.10 T cells proliferated and produced high levels of IL-2 (generally 8,000–20,000 pg/ml), together with undetectable or low levels of IFN-γ (generally less than 2,000 pg/ml) (Fig. 4). In some experiments, IL-2 production was reduced by up to 40% in cultures with CD86 −/− APCs, as compared with wild-type APCs, and occasionally depressed during priming with CD80 −/− APCs. IL-4 production was undetectable by ELISA.

Production of cytokines following secondary stimulation of T cells primed with CD80 −/− or CD86 −/− APCs

The effects of priming in the selective absence of CD80 or CD86 upon T cell differentiation were analyzed following restimulation

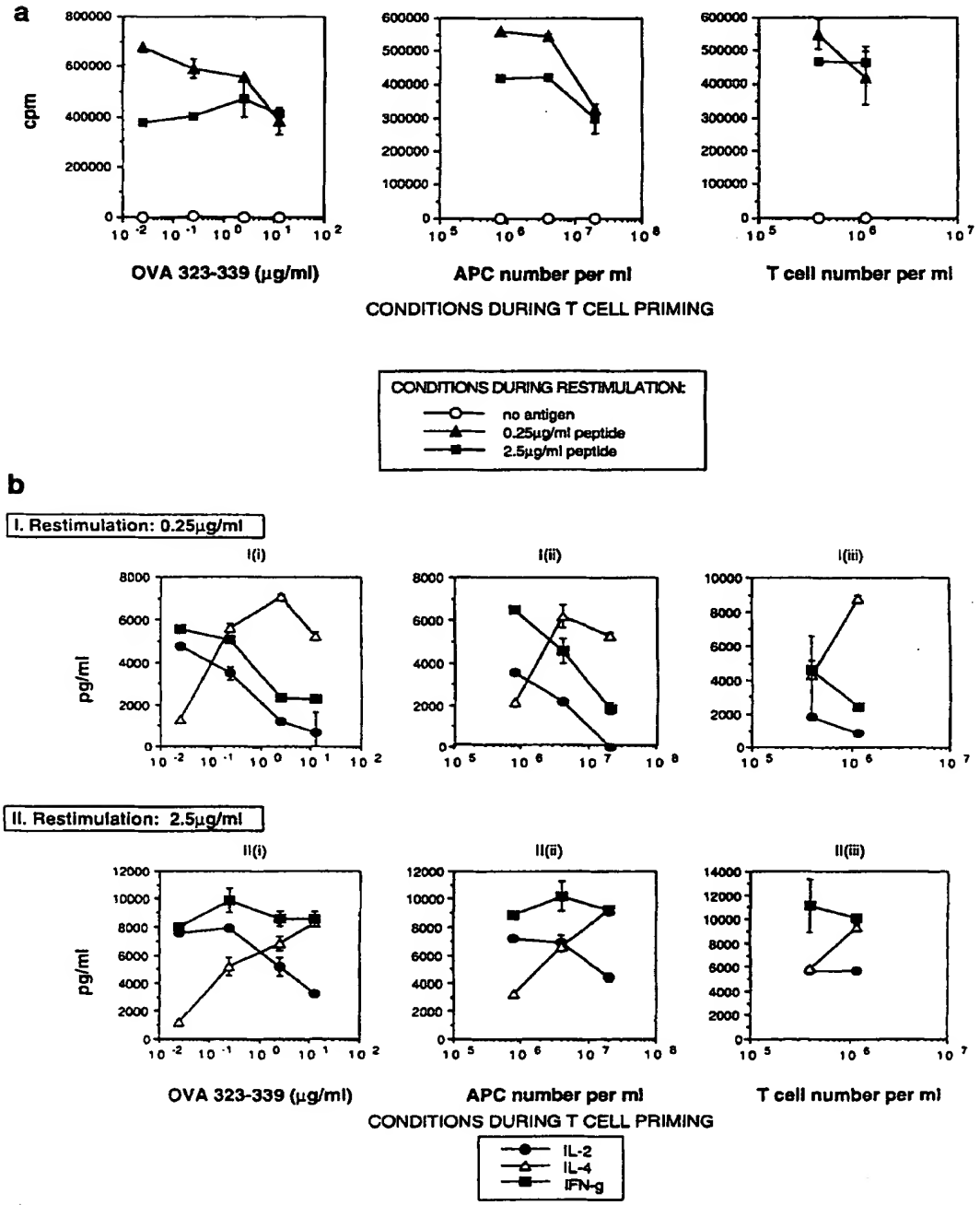

FIGURE 2. Proliferative responses and cytokine production by DO11.10 TCR transgenic T cells during secondary stimulation following priming with a range of peptide concentrations, APC numbers, or T cell numbers. Viable T cells were recovered after 5 days of primary culture (see legend to Fig. 1) by density centrifugation, and rested overnight. T cell numbers were adjusted to 4×10^5 T cells/ml and restimulated in the presence of 0.25 or 2.5 μg/ml $OVA_{323-339}$, as indicated, following addition of an equal volume of a 4×10^6/ml suspension of wild-type T-depleted spleen cells. Proliferation was measured for the final 6 h of a 72-h culture (*a*). Supernatants were collected after 2 days of secondary culture, and levels of cytokine production were measured by ELISA (*b*). In the absence of peptide, no significant cytokine production was detectable during secondary culture. Pairs of figures I(i) and II(i), I(ii) and II(ii), and I(iii) and II(iii) share the same *x*-axis. Cell concentrations in figure and legend refer to cell suspensions before fourfold dilution upon addition to wells. Error bars indicate SE.

in the presence of CD80/CD86-sufficient APCs, to identify the role of CD80 or CD86 during the initial priming stage. Having found that peptide dose, APC number, and T cell number present during priming were capable of dramatically influencing the cytokine profiles of T cells primed in the presence of wild-type APCs, we investigated the influence of a range of conditions on the ability of CD80 −/− and CD86 −/− APCs to prime T cells.

In Figure 5, production of IL-2, IFN-γ, and IL-4 during secondary stimulation is shown for T cells primed by either wild-type, CD80 −/−, or CD86 −/− APCs. We compared the consequences

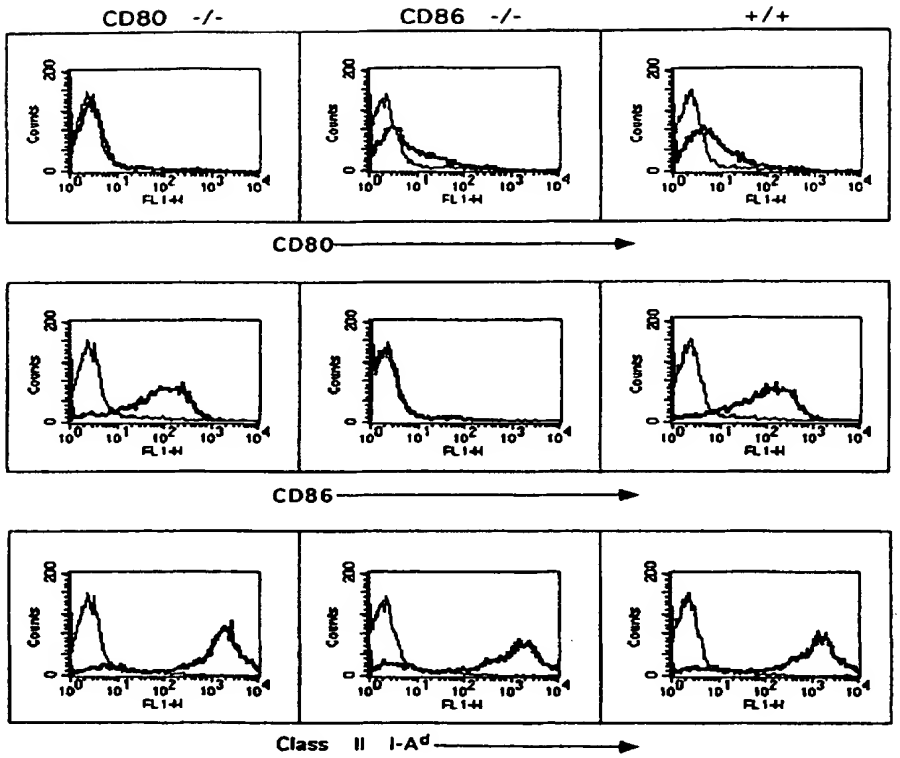

FIGURE 3. FACS analysis of CD80 and CD86 expression on spleen cells obtained from CD80 −/−, CD86 −/−, and wild-type BALB/c mice. Spleen cells recovered from CD80 −/−, CD86 −/−, and wild-type BALB/c (+/+) mice were stimulated at 3×10^6/ml final concentration for 4 days with LPS and dextran sulfate. Viable cells were recovered by density centrifugation, stained for surface expression of CD80 (using clone 1G10), CD86, and class II I-A^d, as indicated (bold line histogram), or a control rat IgG2a (fine line histogram), and analyzed by FACS. The *x*-axis represents $\log_{10}$ fluorescence intensity of staining.

of priming over a 10-fold range of Ag concentrations (Fig. 5*a*), or a 10-fold range of APC numbers (Fig. 5*b*), or a 4-fold range of input T cells (Fig. 5*C*). In each case, it is clear that under suboptimal conditions of T cell priming (e.g., at limiting numbers of APCs or low T cell input), production of IL-4 during secondary culture is abrogated when CD86 −/− APCs are used in the primary culture. Furthermore, if T cells are primed with CD80 −/− APCs, they also produce less IL-4 than after priming with wild-type APCs, although CD86 deficiency generally has a greater effect on priming for IL-4 than CD80 deficiency. When the conditions of T cell priming are optimal or supraoptimal (e.g., with high numbers of APCs in Fig. 5*b*), the T cells make significant levels of IL-4, even if they were primed with CD86 −/− APCs. In some experiments using intermediate APC numbers and high Ag concentrations (conditions similar to those in Fig. 5*a*), or using up to 50 μg/ml Ag even with lower APC numbers, T cells primed with CD86 −/− APCs produced levels of IL-4 similar to (but never 100% of) the level of IL-4 produced by T cells primed with wild-type APCs (data not shown). These results suggest that there is no absolute defect in the ability of CD86 −/− APCs to prime T cells for the production of IL-4.

The levels of IL-2 and IFN-γ produced upon secondary stimulation are also affected by the absence of CD86, and to a lesser extent CD80, during initial priming. However, the relationship between costimulation and cytokine production appears more complex than that for IL-4 production, because the level of Th1 cytokines, particularly IL-2, tends to decrease as peptide concentration, APC, or T cell numbers increase above an optimal level (as also shown in Fig. 2). Nevertheless, under suboptimal conditions of priming, IFN-γ production upon restimulation is reduced substantially, and IL-2 to a lesser degree, when CD86 −/− APCs are used in the primary culture (Fig. 5).

Taken together, these results are consistent with 1) a contribution of CD80 and CD86 to the effective strength of stimulus perceived by the T cell during priming, with CD86 making a greater contribution than CD80; 2) a direct relationship between the level of cytokine production during the primary response and production of IL-4 upon restimulation; and 3) a dependence of secondary IL-4 production upon higher levels of costimulation and/or T cell priming than the levels required to prime for secondary IL-2 and IFN-γ production.

Expression of CD80 and CD86 during primary culture of DO11.10 TCR transgenic lymphocytes with peptide

It has been reported previously (4) that following LPS stimulation, CD86 is expressed earlier and at higher levels than CD80. To determine whether CD86 and CD80 expression differed during antigenic stimulation, we performed bulk cultures of whole spleen and lymph node cells from DO11.10 TCR trangenic mice stimulated with $OVA_{323-339}$. We analyzed expression of CD80 and CD86 on $CD3^-$ cells (predominantly I-A^{d+}, data not shown), daily from the time of culture initiation through 4 days of culture, with three doses of peptide across a 100-fold range (Fig. 6).

CD86 was, indeed, more readily detectable than CD80 at all time points examined. Expression of CD86 was high by day 1 following peptide stimulation, and peaked or plateaued by day 2 (Fig. 6*a*). CD80 expression was barely detectable using either anti-CD80 mAb clone 1G10 (Fig. 6*b*) or clone 16-10A1 (data not shown), which stains LPS/dextran sulfate-stimulated spleen cells with a higher intensity than clone 1G10 (data not shown).

The percentage of $CD3^-$ cells decreased, and $CD3^+$ cells increased with time following all treatments. (Note scale change with time in Fig. 6.) However, the relative change was less in cultures stimulated with lower concentrations of peptide. This presumably reflects the greater expansion of T cells in cultures stimulated with higher peptide concentration.

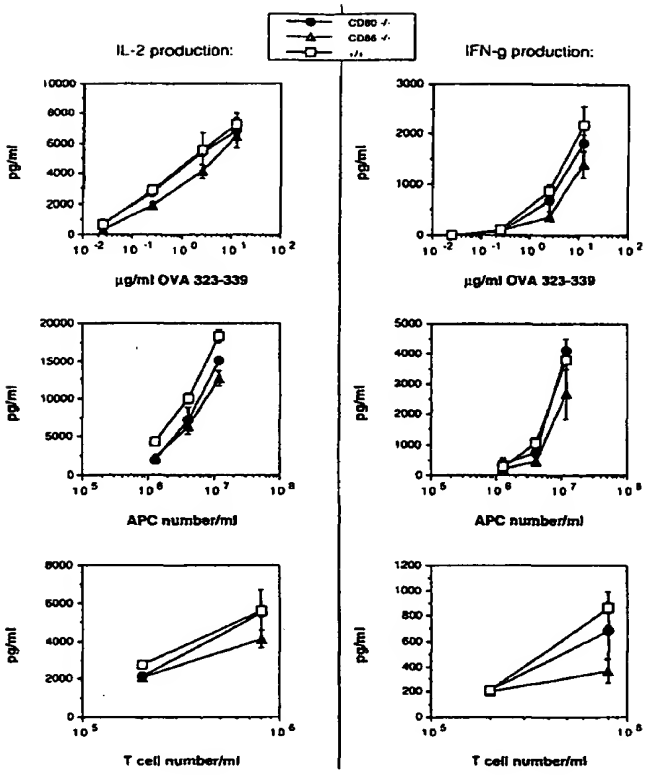

FIGURE 4. Cytokine production during primary stimulation of DO11.10 TCR transgenic T cells in the presence of CD80 −/−, CD86 −/−, and wild-type APCs. T-depleted splenic APCs (4×10^6/ml or as indicated) from CD80 −/−, CD86 −/−, or wild-type BALB/c (+/+) mice were added to an equal volume of a suspension of naive $CD4^+$ T cells (8×10^5/ml or as indicated) from DO11.10 TCR transgenic mice and stimulated with 2.5 μg/ml final concentration of $OVA_{323-339}$ (or as indicated). Supernatants were collected after 2 days of culture, and levels of cytokine production were determined by ELISA. No IL-4 production was detected by ELISA. Cell concentrations in figure and legend refer to cell suspensions before fourfold dilution upon addition to wells. Error bars indicate SE.

Influence of selective blockade of IL-4 during primary stimulation upon cytokine production during secondary stimulation

CD86 has been implicated in selectively facilitating the production of IL-4 (and hence Th2 differentiation) (33, 35). To determine 1) whether production of IFN-γ, as well as IL-4, was being influenced in a quantitative manner by the absence of CD86 molecules, as suggested by the data presented above, and 2) confirm that IL-4 was actually being produced in primary cultures stimulated by CD86 −/− as well as wild-type APCs, we investigated the effect of selective blockade of IL-4 production during primary stimulation with wild-type or CD86 −/− APCs. If CD86 −/− APCs selectively failed to induce IL-4 production, anti-IL-4 mAb treatment during stimulation with wild-type APCs should induce a cytokine profile similar to that observed following priming with CD86 −/− APCs.

These experiments revealed two important results. First, neutralizing IL-4 during T cell priming with wild-type APCs completely blocked subsequent IL-4 production, as expected, but did not inhibit (and slightly enhanced) IL-2 and IFN-γ production at the level of priming shown (Fig. 7). When CD86 −/− APCs were used, IL-4 production upon restimulation was reduced dramatically, as it was following anti-IL-4 mAb treatment during priming with wild-type APCs. However, even IL-2 and IFN-γ production were decreased following priming with CD86 −/− APCs, in contrast to the results obtained using wild-type APCs and anti-IL-4 mAb. Second, and importantly, anti-IL-4 mAb treatment during priming with CD86 −/− APCs caused a modest enhancement of IL-2 and IFN-γ production upon restimulation, presumably by blocking the small amount of IL-4 produced during priming with CD86 −/− APCs. However, production of IFN-γ upon restimulation remained depressed relative to levels observed following priming with wild-type APCs plus anti-IL-4 mAb.

These results point out a clear difference in the functional consequences of CD86 deficiency and IL-4 neutralization: CD86 deficiency affects production of both Th1 and Th2 cytokines, whereas selective elimination of IL-4 abrogates Th2 development while retaining or enhancing Th1 responses. Furthermore, these results indicate that IL-4 was being produced during priming with CD86 −/− as well as wild-type APCs.

Discussion

Studies examining the roles of CD80 and CD86 in Th1 and Th2 differentiation have yielded conflicting results. To address this issue, we have examined the ability of CD80 −/− and CD86 −/− APCs to prime T cells for production of IL-2, IFN-γ, and IL-4 upon secondary stimulation under CD80/CD86-sufficient conditions. Our results suggest that neither CD80 nor CD86 plays an obligatory role in priming for production of a particular cytokine when T cells are primed under optimal or supraoptimal conditions. However, CD80/CD86-mediated costimulation is important for the induction of cytokine synthesis, especially under suboptimal conditions of T cell priming.

We consistently observed a decrease in the level of IFN-γ, as well as IL-4, production during secondary stimulation when the level of primary stimulation with CD86 −/−, and to a lesser extent CD80 −/−, APCs was decreased. Our data suggest that CD86 plays a major role in priming T cells for IL-4 production in particular, but that this role is not obligatory, since it can be overcome by increasing the concentration of peptide or the number of APCs or T cells added to the primary culture (Fig. 5). Furthermore, a lesser, but nevertheless significant contribution to IL-4 by CD80 was revealed under limiting conditions of stimulation, in contrast to previous suggestions of opposing functions for CD80 and CD86 in the induction of cytokine production. If the absence of CD86 (or, indeed, CD80) selectively inhibited the production of IL-4 (33), then a similar profile upon secondary stimulation would be expected following priming with either CD86 −/− APCs or wild-type APCs in the presence of anti-IL-4 mAb. However, whereas anti-IL-4 treatment during primary culture with wild-type APCs abrogated IL-4 production during secondary stimulation, CD86 deficiency led to reduced IL-4 and IFN-γ production even in the presence of anti-IL-4 mAb (Fig. 7). Therefore, our data do not support a simple Th1/Th2-determining role for either CD80 or CD86.

Both IL-4 and IFN-γ production upon secondary stimulation were sensitive to APC or T cell number and to peptide concentration during priming with wild-type as well as CD80 or CD86 −/− APCs (Figs. 2 and 5). The data presented in this work, therefore, are consistent with the idea that CD80/CD86-mediated costimulation serves in a quantitative manner to enhance the strength of stimulus perceived by the responding T cell, and consequently enhance the magnitude of the primary response (proliferation and

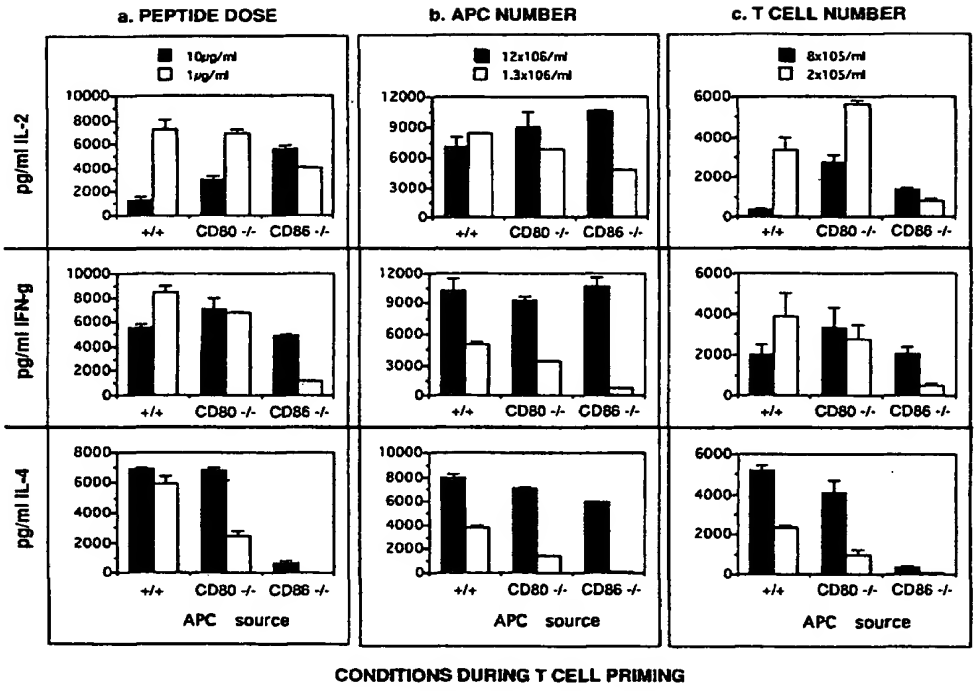

FIGURE 5. Cytokine production during secondary stimulation of DO11.10 TCR transgenic T cells following primary culture with CD80 −/−, CD86 −/−, and wild-type APCs under conditions of high or low level T cell priming. Viable T cells were recovered (see legend to Fig. 2) after 4 days of primary culture under the range of Ag, T cell, and APC concentrations indicated in the figure. Except where stated, a final concentration of 2.5 μg/ml $OVA_{323-339}$ had been used to stimulate primary cultures comprising T cell suspensions at 8×10^5/ml (*a*) or 4×10^5/ml (*b*), and APC suspensions at 4×10^6/ml. Recovered T cells were resuspended to 4×10^5/ml and restimulated with 1 μg/ml $OVA_{323-339}$ following addition of an equal volume of wild-type T-depleted spleen cells (4×10^6/ml). In the absence of peptide, no significant cytokine production was detectable during secondary culture. Levels of cytokine production in supernatants collected after 2 days of secondary culture were measured by ELISA. Cell concentrations mentioned in figure and legend refer to cell suspensions before fourfold dilution upon addition to wells. Error bars indicate SE.

cytokine production) in a manner similar to peptide or MHC concentration. Priming for IFN-γ and IL-2 production is less sensitive than IL-4 to the magnitude of the initial T cell response across the range of stimulus strength investigated. Our data are consistent with previously published reports that IFN-γ production (53) and Th1-type responses (54) are evident at, and peak at, lower levels of antigenic stimulation than IL-4 or Th2-type responses, respectively.

We envisage two possible mechanisms by which CD80 and CD86 molecules might contribute to the level of primary proliferation and cytokine production, and consequently to T cell differentiation. First, the presence or absence of CD80 or CD86 could contribute to the probability with which a T cell will encounter an Ag-specific signal of sufficient strength to trigger activation in an all-or-none manner. Therefore, costimulation would increase the proportion of T cells within the population that become primed for a typical secondary cytokine response. Alternatively, CD80 and CD86 molecules could, in conjunction with the MHC/Ag-specific signal, increase the average strength of signal perceived by a given T cell, such that each responding T cell would be primed to produce a higher level of cytokine. In this case, the strength of signal required for a T cell to produce IFN-γ would be less than that required to produce IL-4. It has been demonstrated recently that provision of B7-mediated costimulation lowers the threshold number of TCRs that must be triggered for a response to be generated by a given dose of Ag (55). Furthermore, in that study, the level of proliferation and/or cytokine production induced rapidly reached a plateau once the threshold TCR occupancy level had been achieved. In the context of our studies, this would imply that for a given dose of peptide, the availability of more costimulatory molecules would enhance the likelihood that a T cell could respond, since fewer TCRs would now have to be triggered. This is consistent with the first possibility outlined above, although effects upon IL-4 production specifically were not investigated in the above-mentioned study. Furthermore, the plateau effect is consistent with the observation that at high levels of stimulation, the absence of CD80 or CD86 plays a minor role; under those conditions, a threshold level of TCR occupancy would be attained regardless of the presence or absence of CD80 or CD86.

The marked contribution of CD86 molecules, and strength of the activating stimulus in general, to T cell differentiation could be explained in terms of the relationship between strength of activating stimulus (and/or strength of response) and the level of IL-4 produced during primary culture. Th2 differentiation occurs via an IL-4-dependent autocrine pathway (56). The higher the concentration of IL-4 within the primary culture, the higher would be the Th2-differentiating capacity of the culture and the relative level of IL-4 production in the secondary culture. We did not detect IL-4 in primary culture supernatants during this series of experiments. However, we assume that low levels of IL-4 are being produced because we were able to block IL-4-driven Th2 differentiation and/or enhance secondary Th1 cytokine production by adding anti-IL-4 mAb during primary culture with both wild-type and CD86 −/− APCs (Fig. 7). Furthermore, DO11.10 TCR transgenic T cells produce low levels of IL-4, detectable by bioassay, when stimulated with CHO cells that have been transfected with class II

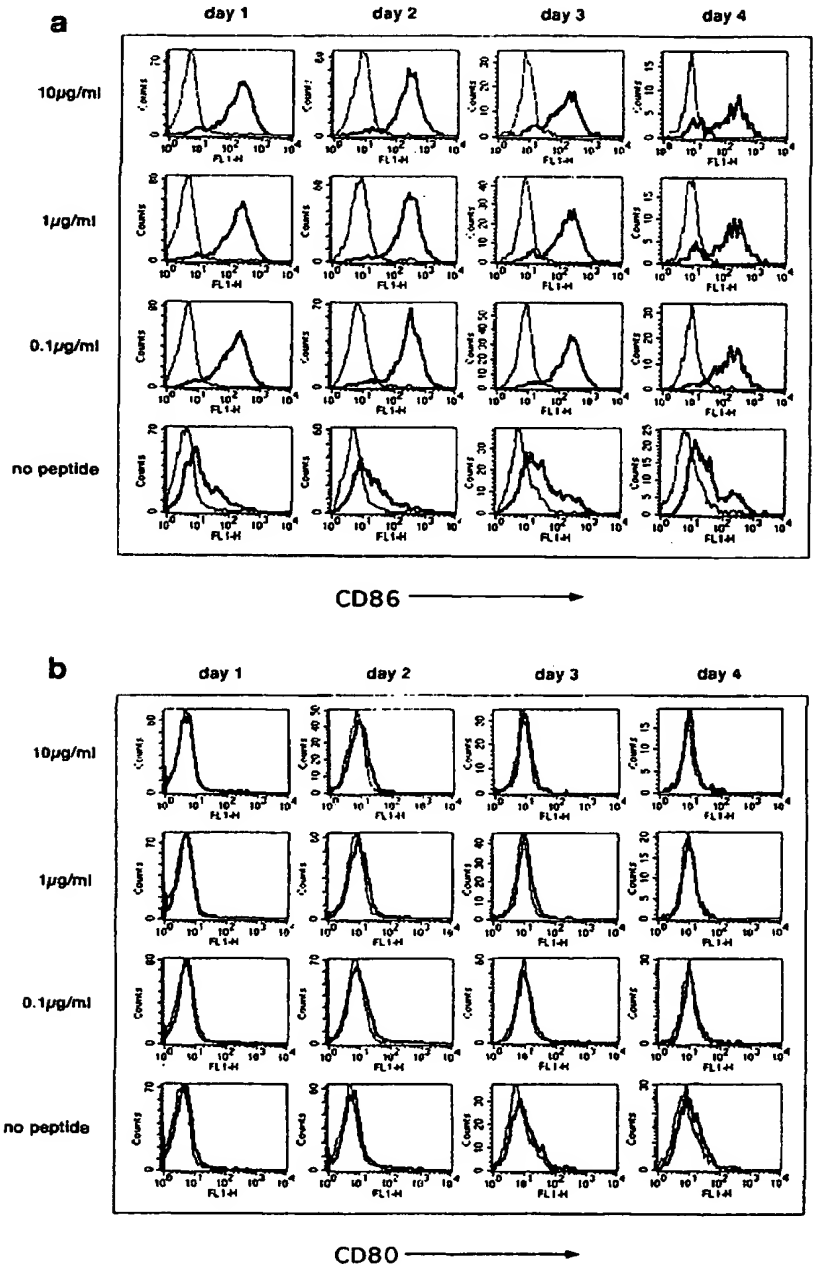

FIGURE 6. FACS analysis of CD80 and CD86 expression following stimulation of DO11.10 TCR transgenic lymphocytes with $OVA_{323-339}$ peptide. Pooled spleen and lymph node cells from DO11.10 TCR transgenic mice were stimulated at 10^6/ml final concentration with 10 μg/ml, 1 μg/ml, or 0.1 μg/ml final concentration of $OVA_{323-339}$, or cultured in the absence of stimulation. Viable cells were recovered daily from day 1 to day 4, as indicated, by density centrifugation. Cells were double stained for co-expression of CD86 or CD80 (clone 1G10) using control rat IgG2a as a control mAb, and CD3, and analyzed by FACS. A gate was drawn around $CD3^-$ cells, and expression of CD86 (bold line histograms, Fig. 6*a*) and CD80 (bold line histograms, Fig. 6*b*) was assessed within the gated population relative to control rat IgG2a (fine line histograms). The *x*-axis represents $\log_{10}$ fluorescence intensity of staining.

I-A^d and CD80 or CD86 molecules (A. N. Schweitzer and R. C. K. Wong, unpublished). The more T cells that became activated and/or the greater the stimulus provided to the T cell, the higher would be the concentration of IL-4 in the primary culture. The autocrine nature of IL-4 production would make the differentiation of IL-4-producing cells particularly sensitive to the initial level of T cell priming, including the contribution made by the presence of CD86. In contrast, the development of Th1 cells is influenced to a large extent by APC-derived stimuli, including IL-12 (57), and may be less dependent on the magnitude of the initial (primary) T cell response.

The greater influence of CD86, as compared with CD80, that we observed upon T cell responses most likely reflects the more rapid up-regulation of CD86 on APCs during cognate interaction with T cells. The level of CD86 expression also appeared markedly higher than that of CD80 during the course of primary antigenic stimulation. Indeed, we detected only minimal expression of CD80 during the 4-day primary culture period using two different anti-CD80 mAbs. However, we cannot rule out the possibility that differences in the affinity and/or level of fluorochrome conjugation between anti-CD80 and anti-CD86 mAbs could be biasing our interpretation of the data. Nevertheless, CD86 expression in the absence of CD80 expression has been similarly observed following Ag-specific activation of B cells in vivo (58) and Ig receptor-mediated activation of B cells in vitro (27).

In many experiments, we still observed a partial effect of CD86 deficiency on IL-4 production, even under circumstances in which the Ag concentration was sufficient to induce near wild-type levels

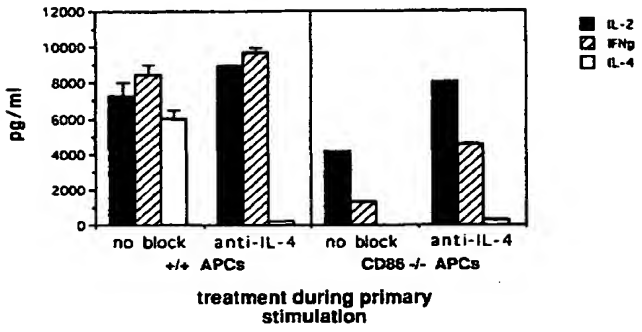

FIGURE 7. The effects of IL-4 neutralization during priming of DO11.10 TCR transgenic T cells with wild-type or CD86 −/− APCs, upon cytokine production during secondary stimulation. Viable T cells were recovered (see legend to Fig. 2) after 4 days of primary culture at 2×10^5/ml final concentration with 1 μg/ml final concentration of $OVA_{323-339}$, in the presence of 10^6/ml final concentration of APCs from wild-type (+/+) or CD86 −/− BALB/c mice, in the presence or absence of an optimally diluted anti-IL-4 hybridoma ascites (11B11). T cells were restimulated at 10^5/ml in the presence of 1 μg/ml $OVA_{323-339}$ and 10^6/ml wild-type APCs. In the absence of peptide, no significant cytokine production was detectable during secondary culture. Levels of cytokine production in supernatants collected after 2 days of secondary culture were measured by ELISA. Error bars indicate SE.

of IL-2 and IFN-γ production. This effect is consistent with a previous study in which it was shown that IL-4 production is preferentially reduced using anti-CD86 mAb (33). In that study, T cells were primed exclusively in the presence of high Ag concentration. Our studies are the first to analyze this question in vitro using a wide range of priming conditions, physiologic APC populations (rather than transfectants), and knockout mice, which provide the definitive approach for establishing the obligatory functions of the CD80/CD86 costimulators. The in vivo correlate of Ag concentration in relation to the APC:T cell ratio is not certain.

Our data concerning the relationship between strength of stimulus, influence on IFN-γ vs IL-4 production, and availability of CD80 or CD86 provide a basis for interpreting the apparently contradictory ability of treatment with anti-CD80 vs anti-CD86 mAb to selectively skew cytokine responses, and the opposing effects that these treatments have in different disease models. Since the production of IL-4 is most sensitive to CD80/CD86 blockade, and since the Th2-promoting effects of IL-4 dominate over the Th1-promoting effects of IL-12 (59), our results predict that initial CD80/CD86 deficiency may prevent Th2 development and could even lead to a relative increase in Th1 responses. Since costimulation is required for sustaining Th1 responses (42–46), CD80/CD86 blockade may also inhibit an ongoing Th1 response. Studies of CD80/CD86 expression and function during in vivo immune responses support these predictions. For example, during EAE, the expression of CD80 and CD86 varies according to the chronicity of stimulation; during the early stages of disease induction, CD86 is the predominant costimulatory molecule detected in the central nervous system, whereas CD80 predominates at later stages (60). Blockade of CD86 from the time of disease induction onward exacerbates disease that has been induced under suboptimal conditions (33), consistent with the idea that IL-4 may be depressing the development of Th1 responses during induction of EAE, and that CD86 is required for priming of Th2 development by virtue of its predominant expression. CD80 appears later, and anti-CD80 suppressed disease (i.e., sustained Th1 activation) in this model. Cessation of anti-CD80 treatment resulted in worsening of the CD80-protected state (33), consistent with a predominant contribution of CD80 to the ongoing stimulation of IFN-γ production. Anti-CD80 and anti-CD86 treatments have the opposite effects upon development of diabetes in the nonobese diabetic mouse model of insulin-dependent diabetes mellitus. This could be explained if the pattern of CD80 and CD86 differed during development, and manifestation of insulin-dependent diabetes mellitus differed from that observed during EAE. Indeed, CD86 expression appears to predominate both before and following pancreatic infiltration (61). Taken together, these results suggest that the influence of CD80 or CD86 on T cell differentiation and effector function is determined mainly by their distinct temporal kinetics and level of expression, rather than distinct signaling capacity of the respective CD80 and CD86 molecules.

Acknowledgments

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EXHIBIT B

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Changes in the strength of co-stimulation through the B7/CD28 pathway alter functional T cell responses to altered peptide ligands

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Abstract

T cells require a TCR and a co-stimulatory signal for activation. We have examined the effect of the strength of TCR and co-stimulatory signals on proliferation and production of cytokines by differentiated T cell clones. The TCR signal was varied using antigen dose and altered peptide ligands. The co-stimulatory signal was varied by using as antigen-presenting cells, Chinese hamster ovary cell transfectants that express different levels of the B7-1 molecule with similar levels of MHC class II. Our results show that the level of co-stimulation has a profound effect on the response to an antigen, and that a strong co-stimulatory signal can convert a weak agonist into a full agonist and an agonist into a superagonist. Antigenicity is not absolute but a function of the strengths of the TCR and co-stimulatory signals. Increasing the strength of co-stimulation can lower antigen concentration required for maximal proliferative responses by T cell clones by 5 log. These results show that the level of expression of co-stimulatory molecules will profoundly regulate T cell clonal expansion and effector functions.

Introduction

Studying the factors that influence $CD4^+$ T cell activation and differentiation is critical to our understanding of the role of these cells in health and disease. The activation of T cells requires at least two signals, an antigenic signal (signal 1) and a co-stimulatory signal (signal 2) delivered by antigen-presenting cells (APC) (1). Antigenic signal alone in the absence of co-stimulation can induce antigen specific unresponsiveness (anergy) (2–5).

A number of groups have attempted to study the parameters that influence T cell activation as well as differentiation. One such approach has been to vary the TCR signal (signal 1) either by altering the antigen concentration (6–8) or by using altered peptide ligands (APL) (9–13). Altering the TCR signal shows that high antigen dose or high-affinity ligand can induce T_h1 cells that secrete IFN-γ and IL-2, whereas low antigen dose or low-affinity ligand can induce T_h2 cells that produce IL-4. We have recently identified an APL that delivers a more potent signal 1 to T cells than the cognate ligand. This APL hyperstimulates T cells in that it induces strong proliferative responses at low antigen dose and also induces secretion of cytokines [IL-2 and tumor necrosis factor (TNF)-α] not observed with cognate ligand (13). Based on these and other studies a concept of 'strength of signal 1' has evolved and has been postulated to play a crucial role in T cell differentiation. However, it is not clear how changes in signal 2 (co-stimulation) will integrate with the strength of signal 1 to affect activation thresholds and effector functions of T lymphocytes.

In this study, we have addressed the role of the strength of co-stimulation (signal 2) in functional responses of T cell clones to cognate and APL. Using as APC, Chinese hamster ovary (CHO) cells that express different levels of B7-1 and a similar level of MHC class II molecules, we show that the level of co-stimulation has a dramatic effect on (i) the antigen

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dose required for maximal proliferative responses of T cell clones, (ii) the secretion of different cytokines and (iii) conversion of a weak/partial agonist to a full agonist.

Methods

Antigens

Proteolipid protein (PLP) (139–151) HSLGKWLGHPDKF (W144) peptide, or its altered forms PLP (A144) HSLGKALGHPDKF, PLP (Q144) HSLGKQLGHPDKF and PLP (A147) HSLGKWLGAPDKF, were synthesized in the laboratory of Dr R. Laursen (Boston University Boston, MA) using FMOC chemistry on a Milligen synthesizer (model 9050; Perspective Biosystems, Framingham, MA).

Antibodies

10.2.16, a mouse anti I-A^k mAb that cross-reacts with I-A^s, was used as the primary antibody for monitoring MHC class II expression on CHO cell transfectants. For detection of B7-1 expression, either FITC-conjugated human CTLA-4–Ig or 1G10, a rat anti- mouse B7-1 antibody, was used. For detection of MHC class II by indirect immunofluorescence either FITC- (Caltag, San Francisco, CA) or phycoerythrin- (Southern Biotechnology Associates, Birmingham, AL) conjugated goat anti-mouse secondary antibody was used. FITC-conjugated goat anti-rat IgG (Caltag) was used as the secondary antibody whenever 1G10 or GL1 was used as a primary antibody for detection of B7-1 or B7-2 expression respectively. When FITC-conjugated CTLA-4–Ig (5) was used for staining cells, negative controls included cells stained with isotype matched FITC conjugated Ig. Results were analyzed on a Coulter flow cytometer (Epics XL-MCL).

Generation of stable transfectants

Full-length I-A^s α and β cDNA clones in Bluescript vector were a gift of Dr S. Miller (Northwestern University, Evanston, IL). The plasmids were digested with *Eco*RI, and the I-A^s α and β chain fragments were isolated, and cloned into an expression vector, SRα (14). Linearized plasmids (25 μg) containing I-A^s α and β chains respectively, along with 5 μg of linearized SV2-Neo-SP65 plasmid were transfected by electroporation into CHO cells, a MHC class II-negative, B7-negative cell line. After selection in medium containing G418 at 400 μg/ml, cells were sorted on a Coulter cell sorter (Epics Elite ESP) for MHC class II expression by indirect immunofluorescence, using 10.2.16 mAb. The sorted cells were >95% positive. The cells were cultured for 4 weeks, resorted and cloned by limiting dilution (0.5 cells/well). Transfectants expressing MHC class II were monitored for stable expression over a period of 4 weeks. One stable transfectant was selected for further transfection. Linearized murine B7-1 plasmid (50 μg) (15) along with 5 μg of linearized pPGK-hygro vector containing the hygromycin-resistance gene were transfected by electroporation into the CHO cell line stably expressing MHC class II. The transfected cells were cultured in selection media containing 400 μg/ml G418 and hygromycin. The antibiotic-resistant cells were sorted for MHC class II and B7 expression using 10.2.16 and FITC-conjugated CTLA-4–Ig. Double-positive cells were cloned by limiting dilution (0.5 cells/well), and were screened for B7-1 and MHC class II expression using 1G10 and 10.2.16 mAb. Clones expressing different levels of B7-1 with similar levels of MHC class II were selected for this study. Cell phenotypes were re-examined before each experiment. All the transfectants were also phenotyped with anti-B7-2 mAb GL1 and found to be negative.

T cell clones

The generation of PLP (139–151)-specific T cell clones ID9 and IE6 has been described elsewhere (16). In brief, ID9 was generated from lymph nodes of SJL mice after immunization with PLP peptide (139–151) in complete Freund's adjuvant (CFA; Difco, Detroit, MI). The lymph node cells were obtained 7–10 days after immunization, stimulated with W144 peptide and propagated in DMEM containing 0.6% T cell growth factor (T-STIM; Collaborative Biomedical Research, Bedford, MA) and 0.06% recombinant mouse IL-2. IE6 was generated by immunizing SJL mice with peptide W144, followed by i.p. administration of monoclonal anti-B7-1 antibody. This clone was expanded and propagated as described above for ID9. Q1.1B6.IE3 (hereafter referred to as 1B6) was generated by immunizing SJL mice with altered peptide Q144 in CFA (13). The clone was expanded and propagated in IL-2 as described earlier (13).

In vitro proliferation assays

CHO cell transfectants were treated with 50 μg/ml mitomycin C (Sigma, St Louis, MO) for 21 h at 37°C. The cells were harvested with 10 mM EDTA in PBS, washed extensively and incubated on ice for ~30 min in media. The cells were washed again 3 times and counted. T cell clones were washed 3 times in DMEM and counted. Mitomycin C-treated CHO cell transfectants (10^4 cells/well) or irradiated (5000 rad) spleen cells (5×10^5 cells/well) and ID9, IE6 or 1B6 T cell clones (10^3–10^4cells/well) were cultured in triplicate in 96-well flat-bottom plates (Becton Dickinson, Lincoln Park, NJ) in the presence of antigen, for 48 h. [^{3}H]Thymidine (1 μCi/well) was added for the last 16 h before harvesting the cells. The [^{3}H]thymidine incorporation was determined in a scintillation counter (model LS 5000; Beckman Instruments) and the result was expressed as mean c.p.m. Supernatants were harvested from the cultures after 40 h for determining the type and amount of cytokines produced. Supernatants were stored at –80°C until assayed.

Cytokine ELISA

Culture supernatants were diluted 1:2 and tested for the presence of cytokines by ELISA as described (16). IL-2, IL-4, IL-10 and TNF-α ELISA components (antibodies and cytokine standards) were obtained from PharMingen (San Diego, CA). In brief, ELISA plates (Immulon 4; Dynatech, Chantilly, Virginia) were coated with purified rat mAb to mouse IL-2 (clone JES6-1A12), IL-4 (clone BVD-1D11), IL10 (clone JES5-2A5) and TNF-α (clone MP6-XT22). Recombinant mouse cytokines IL-2, IL-4, IL-10 and TNF-α were used to construct standard curves. Biotinylated rat mAb to mouse IL-2 (clone JES6-5H4), IL-4 (BVD6-24G2) and IL-10 (SXC-1), and biotinylated polyclonal rabbit anti-mouse TNF-α were used as secondary detecting antibodies. IFN-γ components were obtained

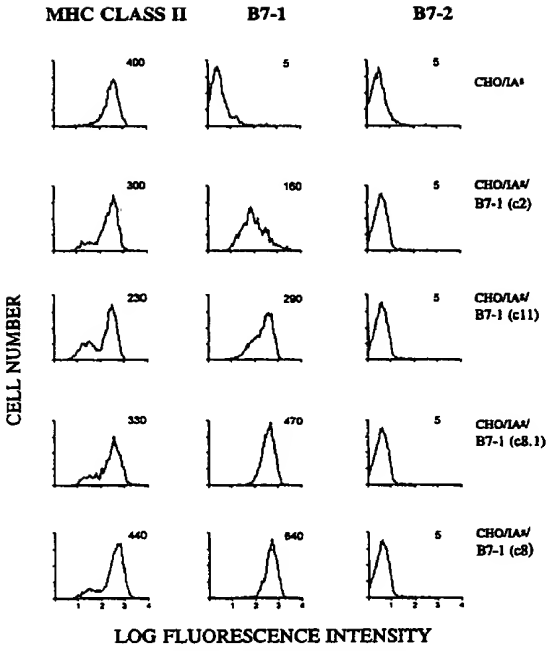

Fig. 1. Expression of I-A^s and B7-1 in CHO cell transfectants. CHO cells were transfected with I-A^s and mouse B7-1 as described in Methods. Controls also included a transfectant that expressed only I-A^s. The expression of MHC class II and B7-1 was determined by 10.2.16 (I-A^s) and 1G10 (B7-1) mAb by indirect immunofluorescence. Negative control included cells stained with anti-B7-2 antibody (GL1) which were found to be identical to cells stained with the secondary antibody alone. MFI is indicated in the upper right of each histogram and the clone description is included on the right.

from Genzyme (Cambridge, MA). Monoclonal hamster anti-mouse IFN-γ was used as the primary antibody, polyclonal goat anti-mouse IFN-γ was used as secondary antibody. Assays were developed in TMB Microwell peroxidase substrate (Kirkegaard & Perry, Gaithersberg, MD) and read at 450 nm after addition of stop solution using a model 3550 Microplate Reader (BioRad, Hercules, CA). Each clone was assayed for all five cytokines and if the data is not presented, this indicates the cytokine was below the level of detection.

Results

Generation of CHO cell transfectants expressing I-A^s and different levels of B7-1

A series of CHO cell transfectants expressing similar levels of I-A^s and varying levels of B7-1 were used as APC in this study (Fig. 1). CHO/I-A^s/B7-1 (clones 2, 11, 8.1 and 8) expressed increasing levels of B7-1, with mean fluorescence intensities (MFI) of 160, 290, 470, and 640 respectively. As control, a transfectant (CHO/I-A^s) that expressed a similar level of MHC class II but no B7 was used. The transfectants were also phenotyped with anti-B7-2 antibody (GL1) and, as expected, all were negative (Fig. 1).

Table 1. Antigenic specificity and cytokine profiles of T cell clones used in this study

T cell clone	Cognate ligand	Cytokine profile	Agonist	Weak agonist
Q1.1B6.IE3	Q144 (HSLGKQLGPDKF)	IFN-γ	None	A144
IE6	W144 (HSLGKWLGPDKF)	IFN-γ, IL-4, IL-10	A147	None
ID9	W144 (HSLGKWLGPDKF)	IFN-γ, IL-2, IL-10	None	A147

The antigen specificities and the cytokine profiles of the T cell clones were defined using cognate ligand and irradiated syngeneic spleen cells as APC. The sequences of the cognate ligands, including the native form of PLP (139–151), W144, and the altered form, Q144, are indicated in the parentheses.

Varying the level of B7-1 expression has a dramatic effect on the antigen dose required for maximal functional responses by T cell clones

Table 1 shows the antigen specificity and cytokine profile of the T cell clones used in this study. The cytokine profiles of these clones were obtained with cognate ligand using irradiated syngeneic spleen cells as APC. APL that act as partial or full agonists for each of the T cell clones are also given in Table 1. To study the interplay of strength of signal 1 with signal 2, we investigated whether differences in strength of co-stimulation could lead to different functional responses with increasing TCR signal. A panel of transfectants expressing different levels of B7-1 with similar levels of MHC class II were used as APC to stimulate a T cell clone, IE6, with its cognate antigen W144. Control APC included irradiated spleen cells or CHO/I-A^s that expressed MHC class II alone. The response was studied over a 7 log range of antigen dose (0.0001–100 μg/ml). Our results demonstrate that the level of co-stimulation had a profound effect on the proliferative response as well as cytokine production by this clone (Fig. 2). The transfectant expressing the highest level of B7-1 [CHO/I-A^s/B7-1 (c8)] shifted the antigen dose–response curve 4 log compared to transfectants that expressed the lowest level of B7-1 [CHO/I-A^s/B7-1 (c2)] or the irradiated syngeneic spleen cells. The proliferative response increased in the order of B7-1 expression, c8 > c8.1 > c11 > c2, and was proportional to B7-1 expression levels. This pattern was also reflected in cytokine production by clone IE6. As the co-stimulatory signal increased, both IFN-γ and IL-10 production required a lower antigen dose. In the absence of co-stimulation, IL-4 was detectable only at the highest antigen dose. IL-4 production was augmented by B7-1 co-stimulation; however, IL-4 production was much less dependent on the level of co-stimulation. Also, IL-4 required a lower antigen dose for maximal production compared to IFN-γ or IL-10 (Fig. 2). The cognate antigen W144 did not induce detectable IL-2 production from the IE6 T cell clone (data not shown). For this clone at the highest antigen doses, irradiated spleen cells consistently induced higher levels of cytokines, particularly IL-4, than the transfectants, probably due to expression of additional accessory molecules on the APC besides B7 (Fig. 2). Thus, these experiments showed that varying the strength

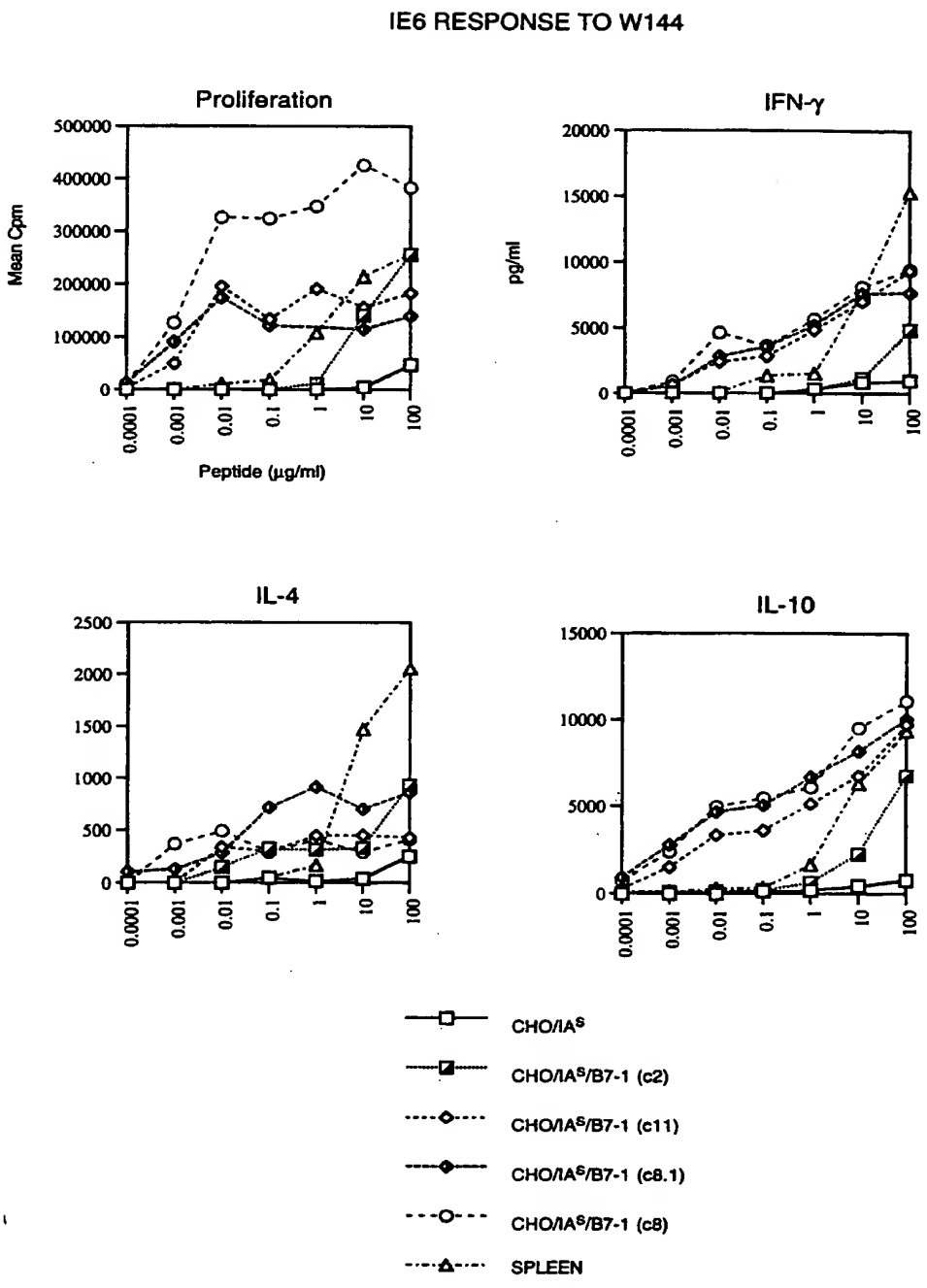

Fig. 2. Proliferative response and cytokine profile of T_h0 clone IE6 using the cognate ligand W144 and a panel of APC. APC included mitomycin C-treated CHO cells expressing similar levels of I-A^s and varying levels of B7-1. A CHO cell transfectant that expressed I-A^s alone and irradiated spleen cells were also used as controls. The functional response was assessed over a wide range of antigen dose (0.0001–100 µg/ml). Proliferation was measured by [^{3}H]thymidine incorporation. Cytokine analysis was done on 40 h culture supernatants by ELISA. The data is representative of two independent experiments.

of co-stimulation had a dramatic effect on shifting the antigen dose required for maximal functional responses of T cell clones.

Higher levels of co-stimulation and agonist ligands hyperstimulate T cell clones and induce production of additional cytokines

To further confirm whether the effects of strength of co-stimulation on functional responses of T cell clones was generalizable, we studied two additional T cell clones with different antigen specificities (Table 1). The strength of co-stimulation was varied as before by varying the level of B7-1 expression. Two transfectants CHO/I-A^s/B7-1 (c11) and CHO/I-A^s/B7-1 (c8) that expressed low and high levels of B7-1 (MFI of 290 and 640 respectively, see Fig. 1 for details) were used as APC. Control APC also included irradiated spleen cells and CHO/I-A^s that expressed MHC class II alone. The T cell clone 1B6, was stimulated with its cognate ligand Q144 using this panel of APC. The high expressor of B7-1 [CHO/I-A^s/B7-1 (c8)] was able to induce a much stronger proliferative response and shifted the antigen dose–response curve 3 log compared to the low B7-1 expressor, CHO/I-A^s/B7-1 (c11), irradiated spleen cells or CHO/I-A^s (Fig. 3a). As before, higher co-stimulation induced higher levels of IFN-γ production at low antigen dose. The clone 1B6 does not produce IL-2 when activated with cognate ligand Q144 and splenic APC even at the maximal antigen concentration tested (13). However, when this clone was activated with cognate ligand and high expressor B7-1 CHO transfectant, there was induction of IL-2. We have observed similar induction of IL-2 from this clone when activated with superagonist ligand L144 (13). Thus high B7-1 expressor CHO cells not only induce heteroclitic proliferative responses and increased production of cytokines when activated with the cognate ligand, they induce production of new cytokines (e.g. IL-2 from 1B6) that are not induced by the cognate ligand with irradiated splenic APC. Thus, these data suggest that increased co-stimulation through B7-1 can change an agonist into a superagonist.

Strength of co-stimulation also had a dramatic effect on the functional response of T cell clone ID9, for which W144 is the cognate ligand. As observed with clone 1B6, when clone ID9 was stimulated with its cognate ligand (W144), antigen presentation by transfectants expressing high levels of B7-1 shifted the antigen dose–response curve 3 log compared to the low B7-1 expressor or irradiated spleen cells (Fig. 3b). Moreover, IL-2 was produced only with higher co-stimulation and antigen dose, and was found to be completely dependent on co-stimulation as it was not produced when CHO/I-A^s or irradiated spleen cells were used as APC (Fig. 3b). Thus the strength of the T cell response is consistently dependent on the strength of the co-stimulatory signal.

Using cognate ligand (agonist) or altered forms of this ligand, we have identified APL that have similar binding affinity for I-A^s but either hypostimulate or hyperstimulate T cell clones (Table 1). These APL provide unique tools that can be used to study the effects of varying the TCR signal on the functional response of T cell clones without affecting MHC loading. The T cell clone, IE6, was stimulated with an APL, A147, using the same panel of APC as before. A147 induces a slightly stronger (heteroclitic) T cell response compared to the cognate ligand (W144) with ~1 log shift in the antigen dose–response curve using splenic APC but this ligand induces qualitatively the same cytokines from this T cell clone (compare Figs 2 and 3c). When clone IE6 was stimulated with APL A147, antigen presentation by transfectants expressing high levels of B7-1 shifted the antigen dose–response curve for proliferation and cytokine production >2 log compared to the low B7-1 expressor or 4 log compared to irradiated spleen cells (Fig. 3c). With strong B7-1 co-stimulation, proliferation was still maximal even at the lowest dose of antigen, 0.0001 μg/ml. T cells and APC without antigen did not induce proliferative responses or cytokine production (data not shown). IL-2, which is not produced by clone IE6 in response to W144, is produced in response to A147 but production required higher strengths of co-stimulation as well as high antigen dose (Fig. 3c). Interestingly, again IL-4 production was least affected by differences in co-stimulation and while co-stimulation augmented IL-4 production, at high antigen dose this T cell clone produced IL-4 without co-stimulation. At the highest antigen dose, irradiated spleen cells were able to induce higher amounts of IL-4 than the transfectants (Fig. 3c). Neither IE6 or ID9 produced detectable TNF-α. Thus, these results show that a high level of co-stimulation can dramatically reduce the antigen dose required for maximal proliferative responses and cytokine production, essentially converting a strong agonist into a superagonist. Moreover, these results also show that IL-4, IL-10, IFN-γ and IL-2 have different co-stimulatory thresholds for production. With increased co-stimulation, there is a qualitatively different cytokine profile, i.e. induction of additional cytokines such as IL-2 that are not seen either with spleen cells or CHO cells that deliver weaker co-stimulatory signals.

High strength of co-stimulation can convert a weak/partial agonist into a full agonist

Since strong co-stimulation could convert a strong agonist into a superagonist, we examined the effect of strong co-stimulation on activation by a weak/partial agonist. We have generated a panel of partial/weak agonists by single amino acid substitution of the cognate ligand (Table 1). A144 is an APL which is a weak agonist for T cell clone 1B6, based on its ability to induce low functional responses compared to the cognate antigen (Q144) using splenic APC (*cf.* Figs 3a and 4a). With splenic APC, the weak agonist A144 can stimulate low level IFN-γ production but no proliferation (Fig. 4a). Interesting differences were observed with stronger co-stimulation. Using the weak agonist A144, proliferation was not observed with CHO cells expressing lower levels of B7-1, MHC class II alone or irradiated spleen cells but transfectants expressing the highest levels of B7-1 were able to stimulate proliferation at high antigen dose (Fig. 4a). Moreover, the proliferative response with high co-stimulation was equivalent to that obtained with the cognate ligand Q144 using splenic APC (*cf.* Figs 3a and 4a). Transfectants expressing the highest level of B7-1 shifted the antigen dose–response curve for IFN-γ production 1 log compared to splenic APC. None of the APC could stimulate IL-2 production by 1B6 in response to A144, suggesting that IL-2 production requires high TCR signal as well as co-stimulation for production. Thus, with high

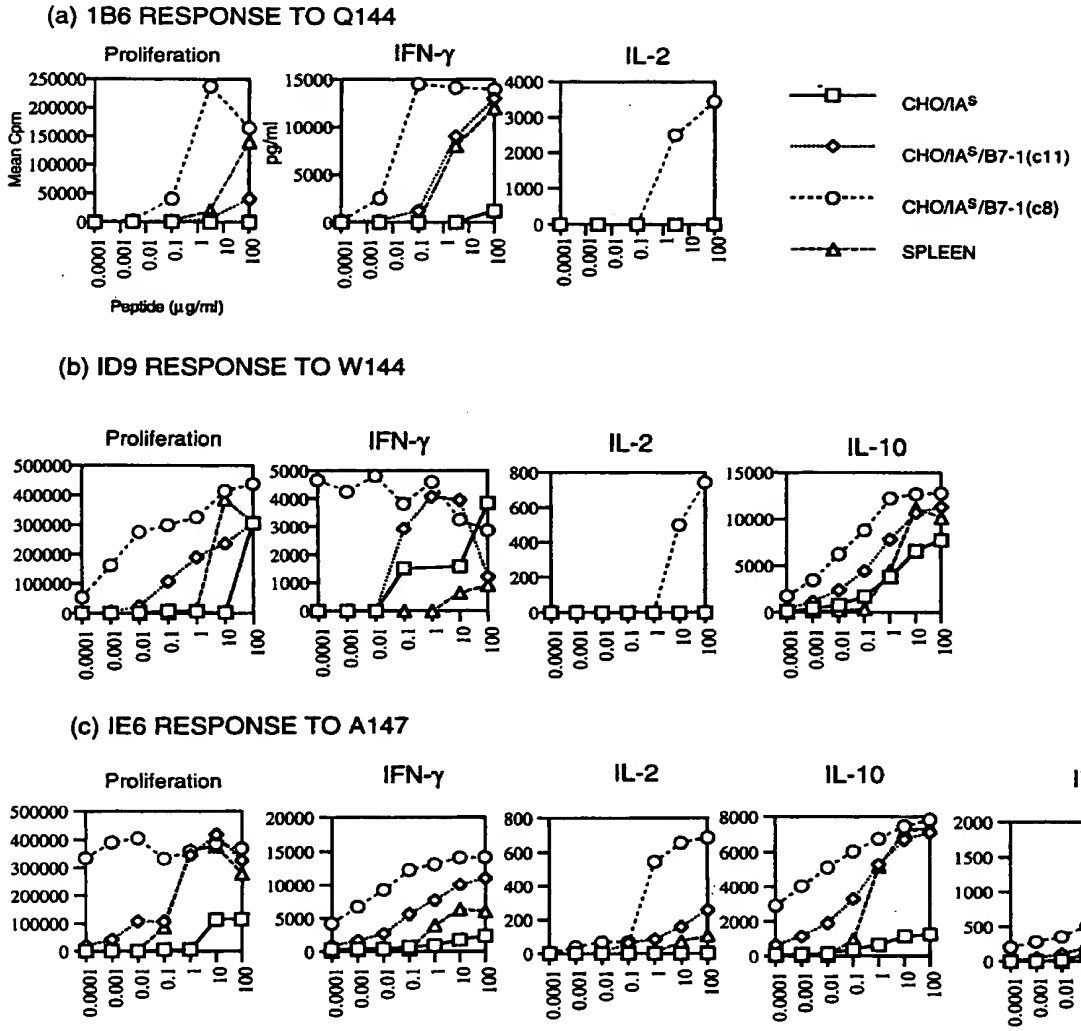

Fig. 3. Proliferation and cytokine production by T cell clones stimulated with agonist ligands using a panel of APC. APC included mitomycin C-treated CHO cells expressing high [CHO/I-A^S/B7-1 (c8)] and low [CHO/I-A^S/B7-1 (c11)] levels of B7-1 with similar levels of class II. Control APC included a CHO cell transfectant that expressed class II alone and irradiated spleen cells. (a) Response of T cell 1B6 stimulated with Q144. (b) Response of T cell clone ID9 stimulated with W144. (c) Response of T cell clone IE6 stimulated with A147. Proliferation was assessed by [^{3}H]thymidine incorporation. Cytokines were measured in 40 h culture supernatants by ELISA. Data representative of two independent experiments.

co-stimulation a weak agonist response could be converted to a full agonist response.

To further confirm that strong co-stimulation can convert a weak agonist into a full agonist, we examined the response of another T cell clone ID9. A147 is an APL which is a weak agonist for T cell clone ID9, based on its ability to induce low functional responses compared to the cognate antigen (W144) using splenic APC (*cf.* Figs 3b and 4b). Clone ID9 was stimulated with A147 using the same panel of APC to study the functional outcome. The highest B7-1 expressor, CHO/I-A^S/B7-1 (c8), was found to be the most effective APC for stimulating the proliferative response as well as cytokine production and at the highest antigen dose the response was equivalent to that observed with the cognate ligand using irradiated spleen cells as APC (*cf.* Figs 3b and 4b). IFN-γ was produced only with high co-stimulation as well as high antigen dose, whereas IL-10 could be produced by other APC providing weaker co-stimulation; however, high co-stimulation induced maximal IL-10 production. This suggests that in this clone, higher strengths of signal 1 and 2 are required for IFN-γ than for IL-10 production. IL-2 was not detectable when clone ID9 was stimulated by A147, suggesting once again

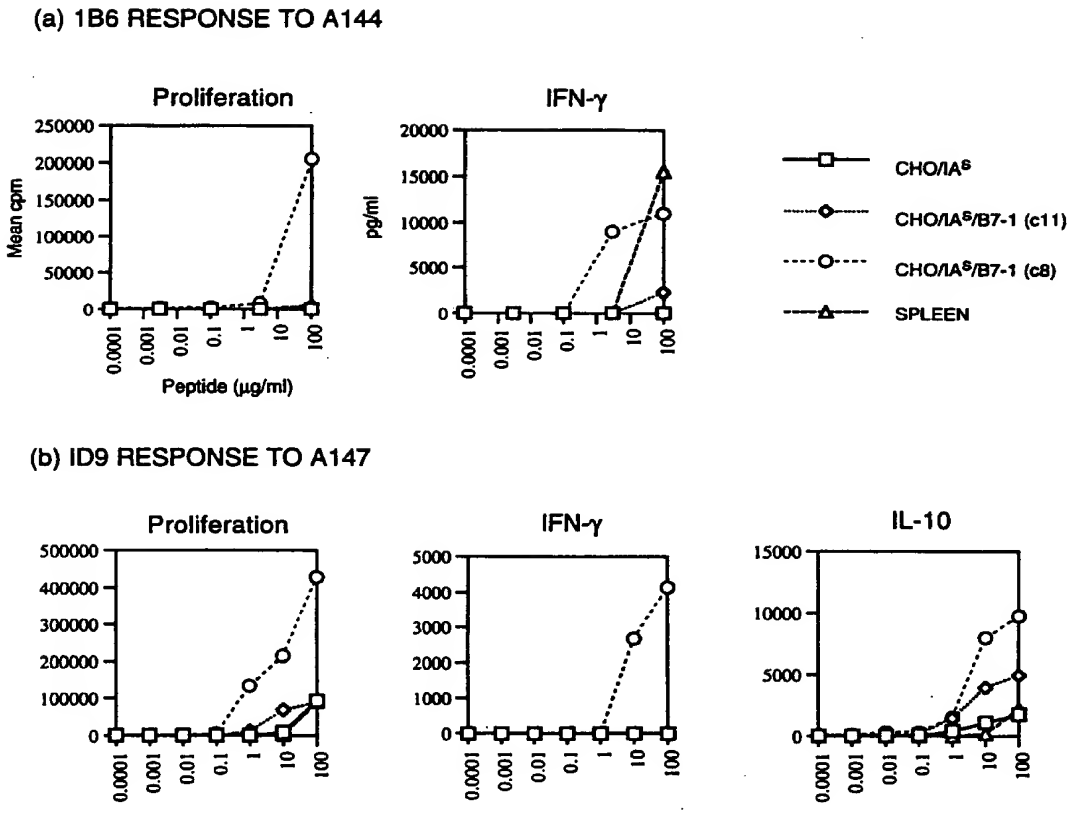

Fig. 4. Proliferation and cytokine production of T cell clones stimulated with weak/partial agonist using a panel of APC. APC included mitomycin C-treated CHO cells expressing high [CHO/I-A^S/B7-1 (c8)] and low [CHO/I-A^S/B7-1 (c11)] levels of B7-1 with similar levels of class II. Control APC included a CHO cell transfectant that expressed class II alone and irradiated spleen cells. (a) T cell clone 1B6 stimulated with a weak agonist A144. (b) ID9 stimulated with a weak agonist A147. Proliferative responses were assessed by [^{3}H]thymidine incorporation and cytokines were measured in 40 h culture supernatants by ELISA. Data representative of two independent experiments.

that a strong TCR signal as well as co-stimulation are essential for IL-2 secretion (Fig. 4b).

Thus, varying the strength of co-stimulation produced a dramatic reduction (2–5 log) in the antigen dose required for proliferation and cytokine production. Higher co-stimulation could convert a weak agonist to a full agonist and induce additional cytokines not observed with the cognate ligand and splenic APC.

Discussion

In this study we have examined the effect of the strength of TCR and co-stimulatory signals on proliferation and production of cytokines by differentiated T cell clones. The TCR signal was varied using antigen dose and APL. The co-stimulatory signal was varied by using as APC, CHO cell transfectants that express different levels of the B7-1 molecule with similar levels of MHC class II. We find that the strength of TCR and co-stimulatory signals determines the T cell response, and discuss the implications for the induction of an autoimmune or anti-tumor response.

The strength of co-stimulation had a profound effect on the antigen dose required for maximal proliferation. At a given strength of TCR signal, we show that proliferation was dependent on the level of co-stimulation. With higher levels of co-stimulation significantly lower antigen dose was required for induction of a strong proliferative response. High levels of co-stimulation could lower the required antigen dose up to 5 log. Our results are consistent with earlier studies, where co-stimulation could lower the antigen dose as well as the number of TCR molecules required for maximal proliferation by T cell clones (17). While a number of studies have shown the importance of co-stimulatory signals or of TCR signals, previous studies have not examined the effect of simultaneously varying TCR and co-stimulatory signals.

The capacity of the strength of co-stimulation to shift the antigen dose–response curve also applies to APL. The response to APL is generally defined using splenic APC. We find that strong co-stimulation can convert a weak/partial agonist into an agonist and convert an agonist into a superagonist. Presentation of a partial agonist with strong co-stimulation can lead to the production of additional cytokines

not seen with splenic APC. As discussed below this result may have an important bearing on the induction of autoimmunity and anti-tumor immunity.

Finally, our study further reveals that each of the cytokines examined, i.e. IL-4, IL-10, IFN-γ and IL-2, has a different co-stimulatory threshold for production that varies depending on the strength of the TCR signal. In differentiated T cell clones, IL-4 production was induced by relatively low co-stimulation and antigen dose and while IL-4 production was augmented by co-stimulation, it was least affected by increases in the strength of co-stimulation. IL-10 and IFN-γ production require intermediate strengths of co-stimulation as well as TCR signal. In contrast, IL-2 production consistently required both higher co-stimulation as well as higher antigen dose (Fig. 3a–c). Thus, the T cell response is not simply on/off but is dictated by the strengths of TCR and co-stimulatory signals.

Previous studies have shown that the strength of the TCR signal dictates cytokine production by naive T cells. In these studies, low dose of antigen induced a T_h2 phenotype, whereas high antigen dose shifted the naive T cells towards T_h1 (6,7). Whether variation in co-stimulation would similarly affect T cell differentiation in naive T cells remains to be determined. Our data are consistent with a number of earlier studies that have altered the strength of the TCR signal by varying the antigen dose (6–8) or using APL (9–13) to show that the T_h1 cytokines, IFN-γ and IL-2, require either high antigen dose or high-affinity ligand for production. A recent study (8) showed that higher strength of TCR signal recruits more cells into the IFN-γ-producing pool, increases the amount of IFN-γ produced per cell and also elicits IL-2 production from these cells. This is consistent with our recent data showing that hyperstimulatory T cell ligands will induce IL-2 and TNF-α from an IL-4/IFN-γ producing T_h0 clone (13). Also, studies where co-stimulation was blocked with CTLA-4–Ig showed a loss of IL-2 secretion, with IL-4 production being unaffected (18). Thus, our findings bring in another dimension and complexity in that we show an interplay between signals 1 and 2 in terms of T cell differentiation, and that the same outcome can be achieved if signal 2 is varied. In addition, we show that when the TCR signal is weak such as with a weak/partial agonist, the strength of co-stimulation then determines the functional outcome of the T cell response in terms of proliferation and cytokine production.

These findings have a direct bearing on the development of autoimmunity where high levels of co-stimulatory molecules might trigger autoreactive T cells. This is supported by studies where the expression of B7 and MHC class II molecules as transgenes on the islets resulted in autoimmune destruction of β cells in the pancreas (19). Furthermore, it has been shown that after activation with IFN-γ, astrocytes express B7 molecules and can prime naive antigen-specific T cells *in vitro* (20). This result suggests that an ongoing inflammatory response in a target organ can induce co-stimulatory molecules such as B7 on non-professional APC, making them capable of activating autoreactive T cells, which would then amplify the autoimmune T cell response and subsequently cause tissue damage by promoting a pro-inflammatory environment. Moreover, recent studies have shown tissue-specific up-regulation of B7-1 in EAE (21). The ability of high strength of co-stimulation to convert a weak agonist into an agonist may allow an infectious agent displaying a cross-reactive epitope (analogous to a weak agonist) to trigger an autoimmune response. If presented by an APC that overexpresses co-stimulatory molecules, the cross-reactive epitope may activate autoreactive T cells that would not respond with only moderate or weak co-stimulation.

In EAE the involvement of T_h1 cells is well documented (22–24), whereas T_h2 cells mediate protection by antagonizing T_h1 cells (25–28). Moreover, adoptive transfer of T cells that are transfected with cDNAs for IL-4 or IL-10 as a transgene has been shown to confer protection (29,30). IL-4 has also been shown to be involved in T cell tolerance induced by aqueous protein antigens *in vivo* (31). This would suggest a possible mechanism by which T_h2 cells can regulate immune responses. Studies carried out in our laboratory have shown that APL can confer protection to EAE by either antagonizing T_h1 responses (32) or by stimulating a subpopulation of T cells that produces T_h2/T_h0 cytokines (10, 28). Moreover, blockade of the CD28/B7-1 pathway has been shown to prevent clinical relapses of murine EAE (33). Our present study shows that the strength of co-stimulation can influence the production of cytokines. Inflammatory cytokines such as IFN-γ require higher strengths of co-stimulation for their secretion. Furthermore, we also demonstrate that higher co-stimulation can convert a weak agonist into a full agonist. This data suggests that cross-reactive analogs of the autoantigen which normally do not stimulate autoreactive T cells may be able to affect T cell differentiation and with high levels of co-stimulation change a protective T_h2 response into a pathogenic T_h1 response. Thus lowering both the TCR and co-stimulatory signals may be necessary for inducing a T_h2 response.

Similarly, transfection of B7 into tumors has been shown by many investigators to lead to tumor rejection and subsequent anti-tumor immunity. Our results would suggest that tumor antigens might be considered to be weak agonists and that the normal immune system does not present tumor antigens with sufficient co-stimulation to activate T cells. Tumor antigens do not come with the associated 'danger' signals common to infectious agents that result in the induction of B7 expression. Presentation of weak tumor antigens by the tumor cell in the context of high co-stimulation may permit the initiation of an anti-tumor response.

In conclusion, studying the role of different parameters that influence T cell activation and differentiation is critical to our understanding of the functional outcome of a T cell response. Such studies may provide invaluable information for shifting a pathogenic response into one that is protective. In the case of an autoreactive T cell repertoire, both the size and the affinity of the repertoire are reduced as only low-affinity T cells that survive negative selection are able to reach the periphery. Since autoantigen is always present, our data would suggest that changes in the level of expression of co-stimulatory molecules such as B7, particularly in the target organ, may play a critical role in activation and expansion of an autoreactive T cell from a protective (T_h0/T_h2) to a pathogenic (T_h1) response and induction of autoimmune disease.

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Abbreviations

APC	antigen-presenting cell
APL	altered peptide ligand
CFA	complete Freund's adjuvant
CHO	Chinese hamster ovary
MFI	mean fluorescence intensity
PLP	proteolipid protein
TNF	tumor necrosis factor

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EXHIBIT C

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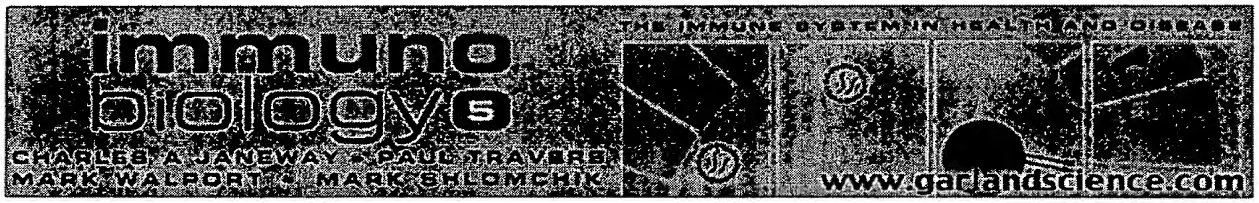

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Immunobiology

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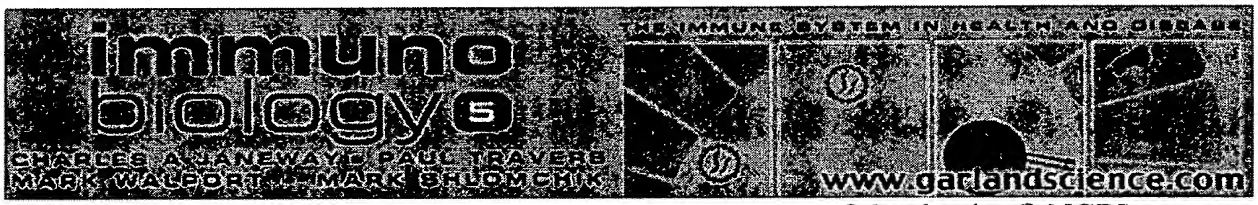

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Part IV. The Adaptive Immune Response
8. T Cell-Mediated Immunity

The production of armed effector T cells

8-5. Both specific ligand and co-stimulatory signals provided by an antigen-presenting cell are required for the clonal expansion of naive T cells.

The best-characterized co-stimulatory molecules are the structurally related glycoproteins B7.1 (CD80) and B7.2 (CD86). We will call them the B7 molecules from here on, as functional differences between the two have yet to be defined. The B7 molecules are found exclusively on the surfaces of cells that can stimulate T-cell proliferation
Although other molecules have been reported to co-stimulate naive T cells, so far only the B7 molecules have been shown definitively to provide costimulatory signals for naive T cells in normal immune responses.
Once a naive T cell is activated, however, it expresses a number of proteins that contribute to sustaining or modifying the co-stimulatory signal that drives clonal expansion and differentiation.
Antigen binding to the T-cell receptor in the absence of co-stimulation not only fails to activate the naïve T cell, it instead leads to a state called anergy, in which the T cell becomes refractory to activation by specific antigen

8-6. Dendritic cells specialize in taking up antigen and activating naive T cells.

The only known function of dendritic cells is to present antigen to T cells, and the mature dendritic cells found in lymphoid tissues are by far the most potent stimulators of naive T cells. This ability is not shared, however, by the immature dendritic cells found under most surface epithelia and in most solid organs. In these tissues, they have an immature phenotype that is associated with low levels of MHC proteins, and they lack co-stimulatory B7 molecules. They are not yet equipped to stimulate naive T cells. When an infection occurs, they are stimulated to migrate via the lymphatics to the local lymphoid tissues, where they have a completely different phenotype.
Although activated mature dendritic cells will also present some self peptides, the T-cell receptor repertoire has been purged in the thymus of receptors that recognize self peptides presented by dendritic cells (see Chapter 7), and thus T-cell responses against ubiquitous self antigens are avoided. In addition, tissue dendritic cells reaching the end of their life-span without having been activated by infection also travel via the lymphatics to local lymphoid tissue. Because they do not express the appropriate costimulatory molecules, these cells induce tolerance to any self antigens derived from peripheral tissues that they display.

The signals that activate tissue dendritic cells to migrate and mature after taking up antigen are clearly of key importance in determining whether an adaptive immune response will be initiated. These signals can be generated through direct interactions with pathogens or by cytokine stimulation, but in both cases they are thought to be a consequence of the recognition of invading pathogens by nonclonotypic receptors of the innate immune system.
In addition to pathogen-associated antigens, dendritic cells are thought to present protein antigens from environmental sources that trigger allergic reactions upon inhalation (see Chapter 12), and alloantigens deriving from a transplanted organ, which form the basis for graft rejection (see Chapter 13). In principle, any nonself antigen will be immunogenic if it is taken up and presented by a dendritic cell that is activated to migrate to nearby lymphoid tissues and mature. The normal physiology of dendritic cells is to migrate, and this is increased by stimuli that activate the linings of the lymphatics, like transplantation, which is why dendritic cells are so potent at stimulating allograft reactions.

8-11. Antigen recognition in the absence of co-stimulation leads to T-cell tolerance.

Antigen recognition in the absence of co-stimulation inactivates naive T cells, inducing a state known as anergy. The most important change in anergic T cells is their inability to produce IL-2. This prevents them from proliferating and differentiating into effector cells when they encounter antigen.

8-12. Proliferating T cells differentiate into armed effector T cells that do not require co-stimulation to act.

Late in the proliferative phase of the T-cell response induced by IL-2, after 4–5 days of rapid growth, activated T cells differentiate into armed effector T cells that can synthesize all the effector molecules required for their specialized functions as helper or cytotoxic T cells. In addition, all classes of armed effector T cells have undergone changes that distinguish them from naive T cells. One of the most critical is in their activation requirements: once a T cell has differentiated into an armed effector cell, encounter with its specific antigen results in immune attack without the need for co-stimulation (Fig. 8.22).

This applies to all classes of armed effector T cells. Its importance is particularly easy to understand in the case of cytotoxic CD8 T cells, which must be able to act on any cell infected with a virus, whether or not the infected cell can express co-stimulatory molecules.

Summary.

The crucial first step in adaptive immunity is the activation of naive antigen-specific T cells by antigen-presenting cells. This occurs in the lymphoid tissues and organs through which naive T cells are constantly passing. The most distinctive feature of antigen-presenting cells is the expression of co-stimulatory molecules, of which the B7.1 and B7.2 molecules are the best characterized. Naive T cells will respond to antigen only when one cell presents both specific antigen to the T-cell receptor and a B7 molecule to CD28, the receptor for B7 on the T cell.

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Part V. The Immune System in Health and Disease
14. Manipulation of the Immune Response

Using the immune response to attack tumors.

Cancer is one of the three leading causes of death in industrialized nations. As treatments for infectious diseases and the prevention of cardiovascular disease continue to improve, and the average life expectancy increases, cancer is likely to become the most common fatal disease in these countries. Cancers are caused by the progressive growth of the progeny of a single transformed cell. Therefore, curing cancer requires that all the malignant cells be removed or destroyed without killing the patient. An attractive way to achieve this would be to induce an immune response against the tumor that would discriminate between the cells of the tumor and their normal cell counterparts. Immunological approaches to the treatment of cancer have been attempted for over a century, with tantalizing but unsustainable results. Experiments in animals have, however, provided evidence for immune responses to tumors and have shown that T cells are a critical mediator of tumor immunity. More recently, advances in our understanding of antigen presentation and the molecules involved in T-cell activation have provided new immunotherapeutic strategies based on a better molecular understanding of the immune response. These are showing some success in animal models and are now being tested in human patients.

14-11. The development of transplantable tumors in mice led to the discovery that mice could mount a protective immune response against tumors.

The finding that tumors could be induced in mice after treatment with chemical carcinogens or irradiation, coupled with the development of inbred strains of mice, made it possible to undertake the key experiments that led to the discovery of immune responses to tumors. These tumors could be transplanted between mice, and the experimental study of tumor rejection has generally been based on the use of such tumors. If these bear MHC molecules foreign to the mice into which they are transplanted, the tumor cells are readily recognized and destroyed by the immune system, a fact that was exploited to develop the first MHC-congenic strains of mice. Specific immunity to tumors must therefore be studied within inbred strains, so that host and tumor can be matched for their MHC type.

Transplantable tumors in mice exhibit a variable pattern of growth when injected into syngeneic recipients. Most tumors grow progressively and eventually kill the host. However, if mice are injected with irradiated tumor cells that cannot grow, they are frequently protected against subsequent injection with a normally lethal dose of viable cells of the same tumor. There seems to be a spectrum of immunogenicity among transplantable tumors: injections of irradiated tumor cells seem to induce varying degrees of protective immunity against a challenge injection of

viable tumor cells at a distant site. These protective effects are not seen in T-cell deficient mice but can be conferred by adoptive transfer of T cells from immune mice, showing the need for T cells to mediate all these effects.

These observations indicate that the tumors express antigenic peptides that can become targets of a tumor-specific T-cell response. The antigens expressed by experimentally induced murine tumors, often termed tumor-specific transplantation antigens (TSTAs), or tumor rejection antigens (TRAs), are usually specific for an individual tumor. Thus immunization with irradiated tumor cells from tumor X protects a syngeneic mouse from challenge with live cells from tumor X but not from challenge with a different syngeneic tumor Y, and vice versa

14-12. T lymphocytes can recognize specific antigens on human tumors.

Tumor rejection antigens are peptides of tumor-cell proteins that are presented to T cells by MHC molecules. These peptides can become the targets of a tumor-specific T-cell response because they are not displayed on the surface of normal cells, at least not at levels sufficient to be recognized by T cells.
Six different categories of tumor rejection antigens can be distinguished.
Although each of these categories of tumor rejection antigen may evoke an anti-tumor response in vitro and in vivo, it is exceptional for such a response to be able to spontaneously eliminate an established tumor. It is the goal of tumor immunotherapy to harness and augment such responses to treat cancer more effectively. In this respect, the spontaneous remission occasionally observed in cases of malignant melanoma and renal cell carcinoma, even when disease is quite advanced, offers hope that this goal is achievable.
Tumor rejection antigens shared between most examples of a tumor, and against which tolerance can be broken, represent candidate antigens for tumor vaccines

14-13. Tumors can escape rejection in many ways.

Burnet called the ability of the immune system to detect tumor cells and destroy them 'immune surveillance.' However, it is difficult to show that tumors are subject to surveillance by the immune system; after all, cancer is a common disease, and most tumors show little evidence of immunological control. The incidence of the common tumors in mice that lack lymphocytes is little different from their incidence in mice with normal immune systems; the same is true for humans deficient in T cells. The major tumor types that occur with increased frequency in immunodeficient mice or humans are virus-associated tumors; immune surveillance thus seems to be critical for control of virus-associated tumors, but the immune system does not normally respond to the novel antigens deriving from the multiple genetic alterations in spontaneous tumors. The goal in the development of anti-cancer vaccines is to break the tolerance of the immune system for antigens expressed mainly or exclusively by the tumor.

It is not surprising that spontaneously arising tumors are rarely rejected by T cells, as in general they probably lack either distinctive antigenic peptides or the adhesion or co-stimulatory molecules needed to elicit a primary T-cell response. Moreover, there are other mechanisms whereby tumors can avoid immune attack or evade it when it occurs. Tumors tend to be genetically unstable and can lose their antigens by mutation; in the event of an immune response,

this instability might generate mutants that can escape the immune response. Some tumors, such as colon and cervical cancers, lose the expression of a particular MHC class I molecule, perhaps through immunoselection by T cells specific for a peptide presented by that MHC class I molecule. In experimental studies, when a tumor loses expression of all MHC class I molecules, it can no longer be recognized by cytotoxic T cells, although it might become susceptible to NK cells. However, tumors that lose only one MHC class I molecule might be able to avoid recognition by specific CD8 cytotoxic T cells while remaining resistant to NK cells, conferring a selective advantage in vivo.

14-14. Monoclonal antibodies against tumor antigens, alone or linked to toxins, can control tumor growth.

The advent of monoclonal antibodies suggested the possibility of targeting and destroying tumors by making antibodies against tumor-specific antigens. This depends on finding a tumor-specific antigen that is a cell-surface molecule. Some of the cell-surface molecules that have been targeted in experimental clinical trials are shown in Fig. 14.18. So far there has been limited success with this approach, although, as an adjunct to other therapies, it holds promise. Some striking initial results have been reported in the treatment of breast cancer with a humanized monoclonal antibody, known as Herceptin, which targets a growth factor receptor, HER-2/neu, that is overexpressed in about a quarter of breast cancer patients.

14-15. Enhancing the immunogenicity of tumors holds promise for cancer therapy.

Although vaccines based on tumor antigens are, in principle, the ideal approach to T cell-mediated cancer immunotherapy, it may be many decades before the dominant tumor antigens for common cancers are identified. Even then, it is not clear how widely the relevant epitopes will be shared between tumors, and peptides of tumor rejection antigens will be presented only by particular MHC alleles. To be effective, a tumor vaccine may therefore need to include a range of tumor antigens.

A further experimental approach to tumor vaccination in mice is to increase the immunogenicity of tumor cells by introducing genes that encode co-stimulatory molecules or cytokines. This is intended to make the tumor itself more immunogenic. A tumor cell transfected with the gene encoding the co-stimulatory molecule B7 (see Section 8-5) is implanted in a syngeneic animal. These B7-positive cells can activate tumor-specific naive T cells to become armed effector T cells able to reject the tumor cells. They are also able to stimulate further proliferation of the armed effector cells that reach the site of implantation. These T cells can then target the tumor cells whether they express B7 or not; this can be shown by reimplanting nontransfected tumor cells, which are also rejected.

The potency of dendritic cells in activating T-cell responses provides the rationale for yet another strategy for vaccinating against tumors. The use of antigen-pulsed autologous dendritic cells to stimulate therapeutically useful cytotoxic T-cell responses to tumors has been developed in experimental models, and there have been initial trials in humans with cancer.

Clinical trials are in progress to determine the safety and efficacy of many of these approaches in human patients. What is uncertain is whether people with established cancers can generate sufficient T-cell responses to eliminate all their tumor cells under circumstances in which any tumor-specific naive T cells might have been rendered tolerant to the tumor. Moreover, there is always the risk that immunogenic transfectants will elicit an autoimmune response against the normal tissue from which the tumor derived.

Summary.

Tumors represent outgrowths of a single abnormal cell, and animal studies have shown that some tumors elicit specific immune responses that suppress their growth. These seem to be directed at MHC-bound peptides derived from antigens that might be mutated, inappropriately expressed, or overexpressed in the tumor cells. T-cell deficient individuals, however, do not develop more tumors than normal individuals. This is probably chiefly because most tumors do not make distinctive antigenic proteins or do not express the co-stimulatory molecules necessary to initiate an adaptive immune response. Tumors also have other ways of avoiding or suppressing immune responses, such as ceasing to express MHC class I molecules, or making immunosuppressive cytokines. Monoclonal antibodies have been developed for tumor immunotherapy by conjugation to toxins or to cytotoxic drugs or radionuclides, which are thereby delivered at high dose specifically to the tumor cells. More recently, attempts have been made to develop vaccines based on tumor cells taken from patients and made immunogenic by the addition of adjuvants, or by pulsing autologous dendritic cells with tumor-cell extracts or tumor antigens. This approach has been extended in animal experiments to transfection of tumor cells with genes encoding co-stimulatory molecules or cytokines that attract and activate dendritic cells.

EXHIBIT D

nature medicine

Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants

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Defects in major histocompatibility complex (MHC) class I–restricted antigen presentation are frequently observed in human cancers and result in escape of tumors from cytotoxic T lymphocyte (CTL) immune surveillance in mice. Here, we show the existence of a unique category of CTLs that can prevent this escape. The CTLs target an alternative repertoire of peptide epitopes that emerge in MHC class I at the surface of cells with impaired function of transporter associated with antigen processing (TAP), tapasin or the proteasome. These peptides, although derived from self antigens such as the commonly expressed Lass5 protein (also known as Trh4), are not presented by normal cells. This explains why they act as immunogenic neoantigens. The newly discovered epitopes can be exploited for immune intervention against processing-deficient tumors through adoptive T-cell transfer or peptide vaccination.

CD8$^+$ CTLs have an important role in the immune defense against cancers[1], and inhibition of MHC class I–restricted antigen presentation is therefore an attractive strategy for tumors to evade immune-mediated destruction[2]. Indeed, impairment of TAP is frequently observed in human cancers[3–5]. TAP is responsible for the delivery into the endoplasmic reticulum (ER) of the cytosolic peptide repertoire resulting from proteolytic breakdown of intracellular proteins, thereby making it available for loading onto MHC class I molecules[6]. The importance of TAP function for the display of peptide epitopes by MHC class I is underscored by the greatly diminished expression of MHC class I molecules at the surface of TAP-deficient cells[7]. This indicates that the vast majority of MHC class I molecules at the surface of processing-proficient cells comprise peptide epitopes of which the delivery to the ER depends on TAP. Accordingly, suppression or loss of TAP function in tumors generally results in failure of recognition and elimination by epitope-specific effector CTLs[8–10].

In our search for immune-effector mechanisms that could counteract the escape of TAP-deficient tumors from CTL-mediated destruction, we have uncovered a category of T cells that is capable of eliminating cells with defects in MHC class I antigen processing. We initially found that immunization of mice with B7.1-expressing, TAP-deficient RMA-S lymphoma cells elicited a CD8$^+$ T-cell response that selectively kills TAP-deficient cells[11]. These findings suggested the existence of a novel CTL target structure and prompted us to investigate this concept in detail. We now show that these T cells are directed against a previously unknown repertoire of peptides that are presented at the surface of processing-deficient cells in the context of both classical and nonclassical MHC class I molecules. Induction of this CTL response, either by adoptive transfer of *ex vivo*–expanded CTLs or by immunization with synthetic peptide epitopes, results in the selective eradication of TAP-deficient tumors *in vivo*.

RESULTS

CD8$^+$ T cells are capable of eliminating TAP-deficient cells

Our previous work indicated the existence of CD8$^+$ T cells capable of reacting against TAP-deficient tumor cells[11]. For an in-depth analysis of these T cells and their target structure (T-cell epitopes associated with impaired peptide processing, TEIPPs), we established long-term T-cell cultures from C57BL/6 (B6) mice by *in vivo* immunization with TAP-deficient RMA-S.B7-1 tumor cells followed by repeated *in vitro* restimulation. The reactivity of these polyclonal T-cell cultures and the clonal T-cell lines derived from them was comparable to that of our previously described CD8$^+$ T cells, in that we observed strong cytolytic activity against RMA-S tumor cells and nontransformed B-cell blasts derived from *Tap1*$^{-/-}$ mice, whereas B-cell blasts of wild-type mice were not lysed (**Fig. 1a–c** and data not shown). More detailed analysis of these T cells by measurement of interferon (IFN)-γ release confirmed their selectivity for TAP-deficient target cells and also

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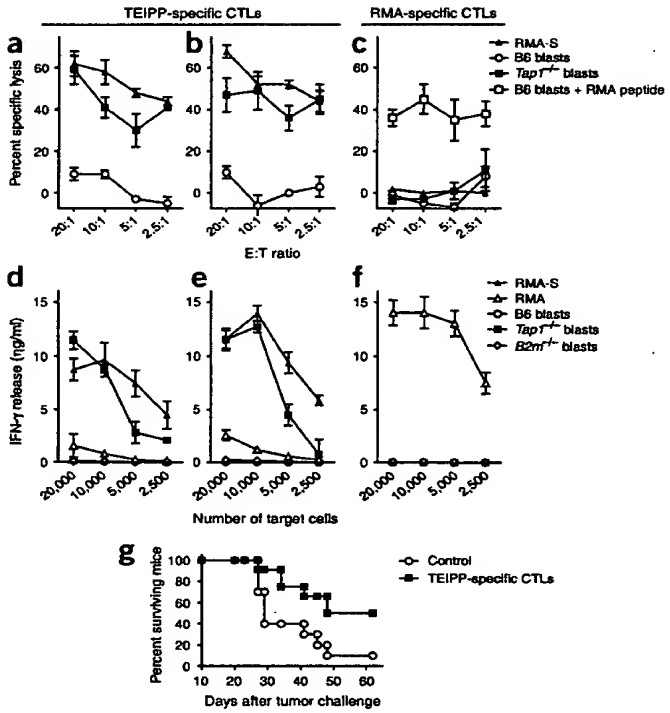

Figure 1 RMA-S.B7-1–induced T cells selectively recognize and eradicate TAP-deficient cells. (**a–f**) The response of two independently derived TEIPP-specific T-cell clones against RMA-S and *Tap1*$^{-/-}$ B-cell blasts was measured in cytotoxicity (**a–c**) and IFN-γ release (**d–f**) assays. Reactivity of the two T-cell clones shown is representative for that of all six available T-cell clones as well as for the >20 independent polyclonal T-cell cultures that we generated. Control CTLs (**c,f**) recognize a TAP-dependent peptide on RMA[30]. (**g**) B6 mice were inoculated with TAP-deficient RMA-S tumor cells. Mice were treated with one of our TEIPP-specific T-cell clones together with IL-2 ($n = 12$). The reactivity of the T-cell clone used is shown in **a** and **d**. Control mice were treated with IL-2 alone ($n = 10$). No changes were observed after the last day depicted. The difference between the two groups was statistically significant (analysis of Kaplan-Meier curves by log rank test, $P = 0.017$). One out of two experiments with similar outcome is shown.

showed that nontransformed B-cell blasts derived from β2-microglobulin (β2m)-deficient mice were not recognized (**Fig. 1d–f**). The requirement of β2m expression suggests that the target structure of TEIPP-specific T cells comprises MHC class I molecules. The data furthermore indicate that this target is present on both transformed and nontransformed cells, provided that these target cells are TAP deficient.

Adoptive transfer of TEIPP-specific T cells into B6 mice that were challenged with a lethal dose of TAP-deficient RMA-S cells showed that these T cells can exert a marked antitumor effect *in vivo*, in that one-half of the treated mice rejected this highly aggressive tumor, whereas the other mice showed clearly delayed tumor outgrowth (**Fig. 1g**). To evaluate the possibility of autoimmune pathology, three mice from the successfully treated group were killed at the end of the experiment and H&E-stained slides from liver, kidney, lung, spleen, intestinal tract and lymph nodes were microscopically examined. This analysis did not show any signs of autoimmune damage (data not shown). This suggests that normal somatic tissues, which are TAP proficient, do not express TEIPPs, allowing selective *in vivo* targeting of the TAP-deficient tumor cells by TEIPP-specific T cells.

Phenotype and function of TEIPP-specific T cells

In view of the unexpected finding that TEIPP-specific T cells, similar to natural killer (NK) cells, selectively recognize targets that express very low surface levels of MHC class I, we analyzed the expression of several CTL and NK-cell markers at the surface of several independently derived T-cell clones. All clones showed a $CD3^+CD4^-CD8^+$T-cell receptor (TCR) α/β^+ phenotype, while in addition expressing several NK-cell markers (**Supplementary Table 1** online). Conventional CTL clones directed against known MHC class I–binding peptides, however, similarly expressed these NK-associated markers (**Supplementary Table 1** online), in accordance with previously published studies[12]. The phenotype of TEIPP-specific T cells was therefore indistinguishable from that of conventional $CD8^+$ CTLs. This also applied to T-cell function, in that reactivity of TEIPP-specific CTLs was strongly inhibited in the presence of CD3-specific or CD8-specific antibodies (**Fig. 2a**). Our combined data indicate that, despite their distinctive reactivity pattern, TEIPP-specific T cells are not different from conventional CTLs and, therefore, may be exploited in immunotherapeutic strategies in a similar manner.

Recognition pattern of TEIPP-specific T cells

Expression of TEIPPs is not restricted to cells of hematopoietic origin, in that a fibrosarcoma tumor cell line (*Tap1*$^{-/-}$MCA) of *Tap1*$^{-/-}$ origin was efficiently recognized by TEIPP-specific CTLs, whereas a fibrosarcoma tumor cell line from a *Tap1*$^{-/-}$*B2m*$^{-/-}$ background did not trigger these CTLs (**Fig. 2b**). Likewise, TEIPP-specific CTLs recognized immortalized mouse embryo fibroblasts of *Tap1*$^{-/-}$ origin (**Fig. 2c**). Furthermore, treatment of MC38 colon carcinoma cells and B16 melanoma cells, TAP-proficient tumors that are otherwise not recognized by TEIPP-specific CTLs, with *Tap1*-specific antisense oligonucleotides resulted in recognition by TEIPP-specific CTLs (**Fig. 2d,e**). These data indicate that the TEIPP target structure is expressed by cells of diverse histological origin, provided that these cells express β2m and have impaired TAP function. This is of interest because TAP deficiencies have primarily been found in human cancers of non-hematopoietic origin[3–5].

We assessed whether targeted interventions in other parts of the MHC class I processing pathway would also result in presentation of the TEIPP target structure. The first step of the MHC class I antigen-processing pathway involves proteasome-mediated degradation of proteins into peptides, which are subsequently transported into the ER by TAP. Inhibition of proteasome activity limits the availability of peptides for TAP transport[13], and thus creates a situation comparable to impaired TAP function. Indeed, treatment of TAP^+ RMA cells with the proteasome inhibitor lactacystin sensitized them for lysis by TEIPP-specific CTLs (**Fig. 2f**). As expected, RMA recognition by control CTLs was decreased (**Fig. 2g**). A deficiency in tapasin, a chaperone protein involved in TAP-mediated peptide loading of MHC class I molecules[14], also resulted in recognition of target cells by TEIPP-specific CTLs (**Fig. 2h**). This recognition could be inhibited by antibodies specific for CD8, supporting the involvement of the TCR in target recognition.

In conclusion, deficiencies at different levels of the MHC class I antigen-processing pathway result in the sensitization of cells of diverse histological origin to recognition by TEIPP-specific CTLs.

Target-cell recognition is MHC class I restricted

Although MHC class I molecules are expressed at very low levels on antigen processing–deficient cells, the requirement for expression of β2m by target cells (**Fig. 1a–f** and **2b**) suggested that TEIPP-specific

CTLs were MHC class I restricted. This condition was investigated with the use of EC7.1 cells, an MHC class I–negative variant of the TAP-deficient RMA-S cells[15]. Testing of independently derived TEIPP-specific CTL clones against a panel of EC7.1 transfectants expressing defined classical and nonclassical MHC class I genes showed that the CTLs, which exhibit otherwise indistinguishable specificities and phenotypes (**Figs. 1** and **2** and **Supplementary Table 1** online), showed distinct MHC restriction patterns. Some TEIPP-specific CTL clones (**Fig. 3a–c**) showed reactivity against target cells positive for K^b, D^b or Qa-1^b, respectively. These experiments showed that of our six CTL clones tested, three were K^b restricted, two were D^b restricted and one was Qa-1^b restricted. For the K^b- and D^b-restricted TEIPP-specific CTLs, comparable results were obtained using K^b and D^b transfectants of a TAP- and class I–negative variant of the B16 melanoma B78H1 (ref. 16; **Fig. 3d,e**). Notably, restoration of TAP function in these cells by *Tap2* gene transfer or by treatment with IFN-γ led to a decreased recognition by TEIPP-specific CTLs, whereas a combination of *Tap2* gene transfer and treatment with IFN-γ abolished recognition. The partial suppression of CTL recognition by *Tap2* gene transfer alone is in accordance with the fact that B78H1 cells are also impaired in expression of several proteasome subunits, a defect that can be restored by treatment with IFN-γ[16], and with our finding that inhibition of proteasome activity results in presentation of the TEIPP target structure (**Fig. 2f**). In conclusion, the TEIPP-specific CTL repertoire seems to comprise diverse specificities that are restricted by different classical and nonclassical MHC class I molecules.

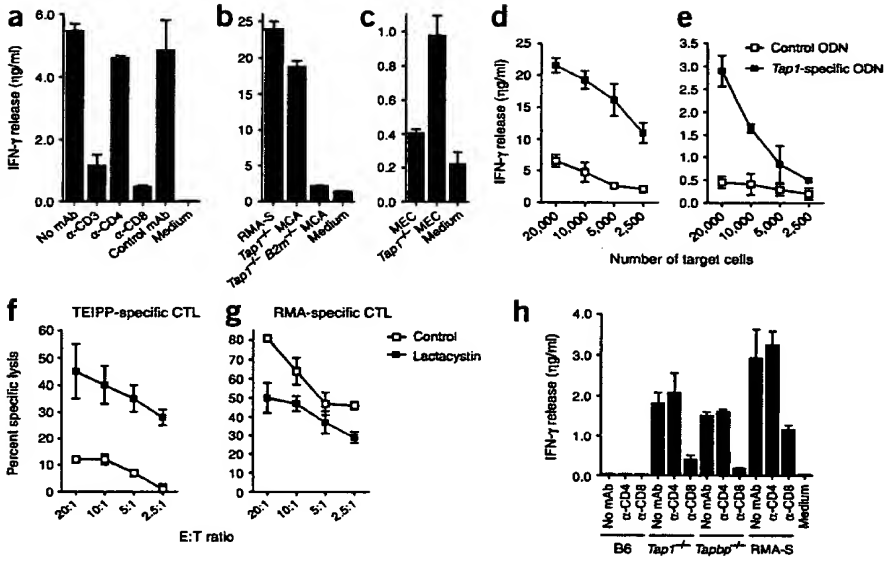

Figure 2 Processing impairment at different stages results in presentation of TEIPPs on cells of diverse tissue origin. (a) TEIPP-specific CTLs were incubated with monoclonal antibodies (mAb) against CD3, CD4 or CD8 before incubation with RMA-S target cells, in order to block TCR signaling. (b,c) TEIPP-specific CTLs were incubated with fibrosarcoma cells (MCA) from *Tap1*$^{-/-}$ or *Tap1*$^{-/-}$*B2m*$^{-/-}$ mice (b), or mouse embryo cells (MEC) from *Tap1*$^{-/-}$ or wild-type mice (c). (d,e) TAP-positive colon carcinoma MC38 (d) and melanoma B16 (e) were loaded with oligo DNA comprising an antisense sequence of *Tap1* (*Tap1*-specific ODN) or control oligo DNA (control ODN). (f,g) TAP-positive RMA tumor cells were treated with the proteasome inhibitor lactacystin and used as targets for cytolysis by TEIPP-specific CTLs (f) or control RMA-specific CTLs (g). Similar results were obtained with the proteasome inhibitors NLVS and LLnL (data not shown). E:T ratio, effector/target ratio. (h) *Tapbp*$^{-/-}$ B-cell blasts are recognized by TEIPP-specific CTLs in a CD8-dependent manner. Spleens of *Tap1*$^{-/-}$, *Tapbp*$^{-/-}$ or wild-type B6 mice were cultured with lipopolysaccharide and used as targets for TEIPP-specific CTLs. Reactivities shown in these experiments are representative of the available TEIPP-specific CTL clones. *Tapbp* encodes tapasin.

Target-cell recognition is peptide dependent

The K^b- and D^b-restricted TEIPP-specific CTLs did not recognize human TAP-deficient T2 cells expressing K^b or D^b (**Fig. 4a,b**). This suggests that recognition by TEIPP-specific CTLs depends on peptides derived from mouse proteins that are not sufficiently conserved between mouse and human. We found that complex peptide libraries based on the K^b or D^b binding motifs selectively sensitized T2 cells for recognition by the respective TEIPP-specific CTLs, providing direct proof for the recognition of peptides by the K^b- and D^b-restricted CTL clones (**Fig. 4a,b**).

These results imply that the processing-deficient cells recognized by our CTLs present a unique, thus far unexplored repertoire of peptide antigens. Because unraveling the molecular nature of the TEIPP peptide repertoire would provide fundamental insight into the basis of their immunogenicity and, moreover, would permit the development of peptide-based vaccine modalities for the treatment of 'escaped' tumors, we eluted naturally processed peptides from affinity-purified K^b and D^b molecules from RMA-S cells. We observed reactivity of TEIPP-specific CTLs against distinct fractions of high-performance liquid chromatography (HPLC)-separated peptides (**Fig. 4c,d**). Similar results were obtained for K^b-restricted CTLs (**Supplementary Fig. 1** online). K^b-eluted peptides did not sensitize D^b-expressing target cells and vice versa (**Supplementary Fig. 1** online), indicating that these MHC class I molecules harbor distinct peptide repertoires. Mass spectrometry analysis of HPLC peptide fractions indicated at least 40 different peptide masses that were selectively present in the CTL-recognized fractions. Although the yield of peptides from large quantities of RMA-S cells (10^{11}) was very low (on average 2 fmol per component), the sequences of the three most abundant peptides from K^b were identified by tandem mass spectrometry (**Supplementary Table 2** and **Supplementary Fig. 2** online). Corresponding synthetic peptides, however, were not recognized by the available K^b-restricted TEIPP-specific CTLs (data not shown), indicating that these three peptides did not constitute TEIPPs for our CTLs. Recovery of D^b-eluted peptides was too low, and the diversity of peptide masses too large, to permit systematic identification of peptide sequences.

Identification of a D^b-presented TEIPP

In view of the technical difficulties described above, we pursued the identification of TEIPPs with a new approach that combined screening of synthetic peptide libraries with information from eluted natural peptides and with database search. We used a D^b-restricted TEIPP-specific CTL clone to screen a synthetic bead–assisted peptide library with a complexity of approximately 6.5×10^5 different 9-mer peptides. This screen resulted in the identification of the mimotope peptide SLSRLSGTV. We then synthesized a collection of 171 single

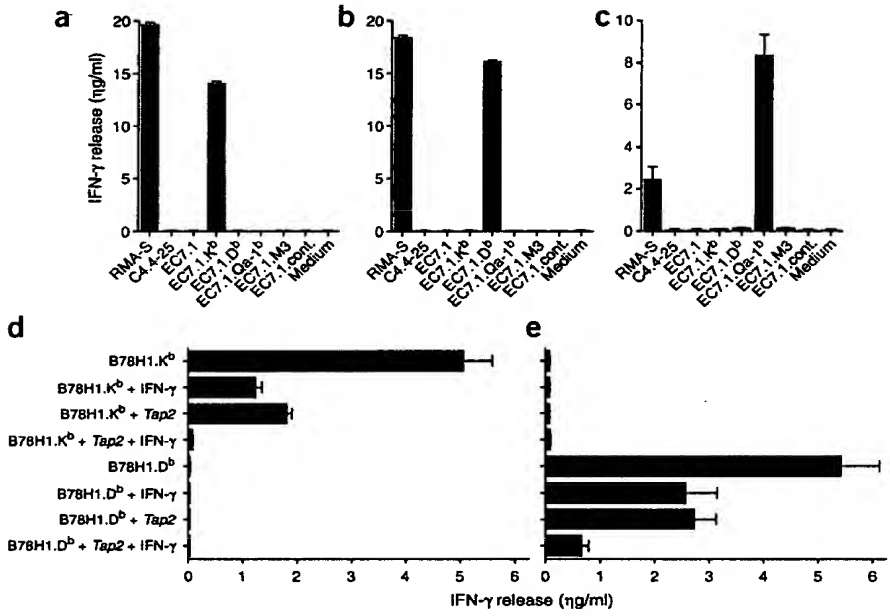

Figure 3 TEIPP-specific CTL reactivity is MHC class I restricted. (a–c) EC7.1 cells, which are MHC class I loss variants of RMA-S[15], were transfected with single classical or nonclassical MHC class I genes (K^b, D^b, $Qa\text{-}1^b$ and M3). C4.4-25$^-$ cells are $\beta 2m^-$ lymphoma cells. (d,e) MHC class I–negative B78H1 melanoma cells[16] were transfected with genes for *Tap2*, *H-2K^b* and/or *H-2D^b* and tested for recognition by K^b- (d) and D^b-restricted TEIPP-specific CTLs (e). Where indicated, cells were treated with IFN-γ. One representative experiment of three is shown. Of note, the response of K^b- and D^b-restricted CTL clones against RMA-S could be blocked in the presence of K^b- and D^b-specific monoclonal antibodies, respectively (data not shown).

peptides in which each position of this mimotope sequence was exchanged for all other amino acids. Measurement of CTL reactivity against this peptide collection showed that some exchanges completely abolished recognition, whereas others strongly improved the recognition (**Supplementary Table 3** online). All amino acid substitutions that led to equal or improved CTL recognition were combined to formulate a 'TCR motif pattern' (**Table 1**). Pattern search in the EMBL mouse protein index database yielded 77 naturally occurring 9-mer peptides that matched this motif. To determine which of these 77 candidates constituted the naturally processed epitope recognized by our D^b-restricted CTLs, we applied three selection criteria (**Supplementary Table 4** online). First, the peptides should trigger the CTLs at low concentrations. Of the 77 tested peptides, we found that 13 elicited CTL activity at concentrations of 250 pM or lower. Secondly, the retention time on HPLC of the candidate peptides should match that of the naturally eluted peptide. This criterion was fulfilled by 5 of the 13 remaining candidates. Finally, the cDNAs encoding these five peptides were cloned and transfected into TAP-inhibited recipient cells to examine proper processing and presentation to CTLs. Only one cDNA conferred CTL recognition: the *Lass5* gene (also known as *Trh4*), encoding an ER-membrane spanning protein that is a member of the TRAM, LAG1 and CLN8 (TLC) family of fatty-acid regulators[17,18] (**Supplementary Fig. 3** online). Transfection of *Lass5* cDNA into TAP-inhibited cells resulted in stronger CTL recognition than transfection into TAP-proficient counterparts (**Supplementary Fig. 3** online), supporting the idea that the encoded CTL epitope is more efficiently presented when TAP function is impaired.

The conclusion that *Lass5* encodes the natural epitope of the D^b-restricted TEIPP-specific CTL clone is based on the following five findings. Firstly, transfection of *Lass5* cDNA selectively sensitized D^b-expressing targets for CTL recognition (**Fig. 4e**). Secondly, the 9-mer peptide MCLRMTAVM derived from Lass5 protein is efficiently recognized by the D^b-restricted TEIPP-specific CTLs, at much lower concentration than the mimotope peptide that was selected from the synthetic peptide library (**Fig. 4f**). Thirdly, the HPLC retention time of the MCLRMTAVM peptide matched that of the natural D^b-restricted epitope as eluted from RMA-S (**Fig. 4g**). Fourthly, the acquired knowledge of the peptide sequence and its corresponding mass allowed us to trace this peptide in the HPLC fraction that was recognized by the D^b-restricted CTLs. The fragmentation spectrum of this mass (MH^+ 1,055.49 Da), as obtained by tandem mass spectrometry, matched exactly with that of the synthetic peptide MCLRMTAVM (**Supplementary Fig. 4** online). The fifth crucial piece of data was the position of the MCLRMTAVM peptide in the Lass5 protein. GenBank lists two alternatively spliced *Lass5* gene transcripts (**Fig. 5a**). Inclusion of exon 9a into the longer *Lass5* transcript leads to a frameshift and early termination of translation in exon 10 (**Fig. 5b**). The key consequence of this frameshift is that the resulting protein comprises the peptide MCLRMTAVM as its carboxy terminus. Because the C-terminal domain of the Lass5 protein protrudes into the ER lumen[17], the processing of this peptide is expected to be independent from proteasome activity and TAP activity[19].

Detection of *Lass5* mRNA expression by RT-PCR showed that both splice variants are widely expressed in transformed and nontransformed cells, in line with the general metabolic function of the *Lass5* gene products (**Fig. 5c**). Notably, the TEIPP-encoding long *Lass5* transcript is expressed by both TAP-proficient and TAP-deficient cells. Furthermore, binding of the MCLRMTAVM peptide to H-2D^b is comparable to that of several known TAP-dependent, D^b-restricted CTL epitopes (**Supplementary Fig. 3** online). It is therefore conceivable that this epitope is available in the ER of all cells, but that only in processing-deficient cells does it gain access to MHC class I molecules because of the absence of an abundance of TAP-dependent peptides.

Peptide vaccination induces protective antitumor CTLs

Vaccination with synthetic peptides comprising tumor-specific T-cell epitopes have been shown to induce protective antitumor immunity in various mouse tumor models[1,20]. We therefore immunized groups of mice with the newly identified D^b-restricted TEIPP-specific CTL epitope, or a control CTL epitope, in combination with a previously identified tumor-specific $CD4^+$ T-helper epitope[21], and challenged the mice with a lethal dose of RMA-S cells. Naive mice and mice that were immunized with control peptide developed progressively growing tumors, whereas immunization with the Lass5-derived peptide prevented tumor outgrowth in the majority of mice (**Fig. 5d**). These data show that TEIPPs may be applied effectively in peptide-based vaccines for CTL-mediated targeting of processing-deficient tumors.

DISCUSSION

Deficiencies in MHC class I antigen presentation are known to provide target cells with an opportunity to evade CTL-mediated killing. Therefore, the discovery of CTLs with a clear preference for target cells with impairments in antigen processing initially presented us with a paradox. The identification of the peptide MCLRMTAVM as the natural epitope recognized by one of these CTLs now sheds light on this issue and provides insights into a novel repertoire of immunogenic peptides that are displayed on the surface of processing-deficient cells. Although this epitope is derived from the commonly expressed self protein Lass5, the immune system considers it as a neoantigen because of its apparent absence from the surface of normal, TAP-proficient cells.

In our search for the mechanism explaining the absence of this epitope on processing-proficient cells, we noted that the *Lass5* gene is expressed at similar levels by processing-deficient and processing-proficient cells. Furthermore, binding of this peptide to H-2D^b is comparable to that of several known TAP-dependent, D^b-restricted CTL epitopes. Accordingly, low quantities of this peptide were sufficient to sensitize target cells for CTL recognition, it could be identified in eluates from affinity-purified D^b molecules and was effective in inducing CTL immunity in peptide-immunized mice. These circumstances set it apart from the poorly binding peptides comprised by peptide-MHC complexes that appear at the surface of RMA-S cells when these cells are grown at lower temperatures, because these complexes quickly fall apart when the cells are transferred to 37 °C[22]. In view of these findings, the failure of the Lass5 peptide to be loaded into MHC class I molecules of processing-proficient cells is most readily explained by the presence in the ER of only very limited quantities of this peptide as compared to the overwhelming amounts of competing peptides that are pumped into the ER by TAP[23]. In the case of the identified Lass5-derived peptide, these limitations could be the result of the low expression of the *Lass5* gene product concerned, as it seems to be encoded by a minor mRNA splice variant, and/or the inefficient generation of this peptide through proteolytic degradation of the Lass5 protein. Because normal somatic cells are processing proficient, the failure to compete for MHC class I binding with the TAP-dependent peptide repertoire can account for the fact that the

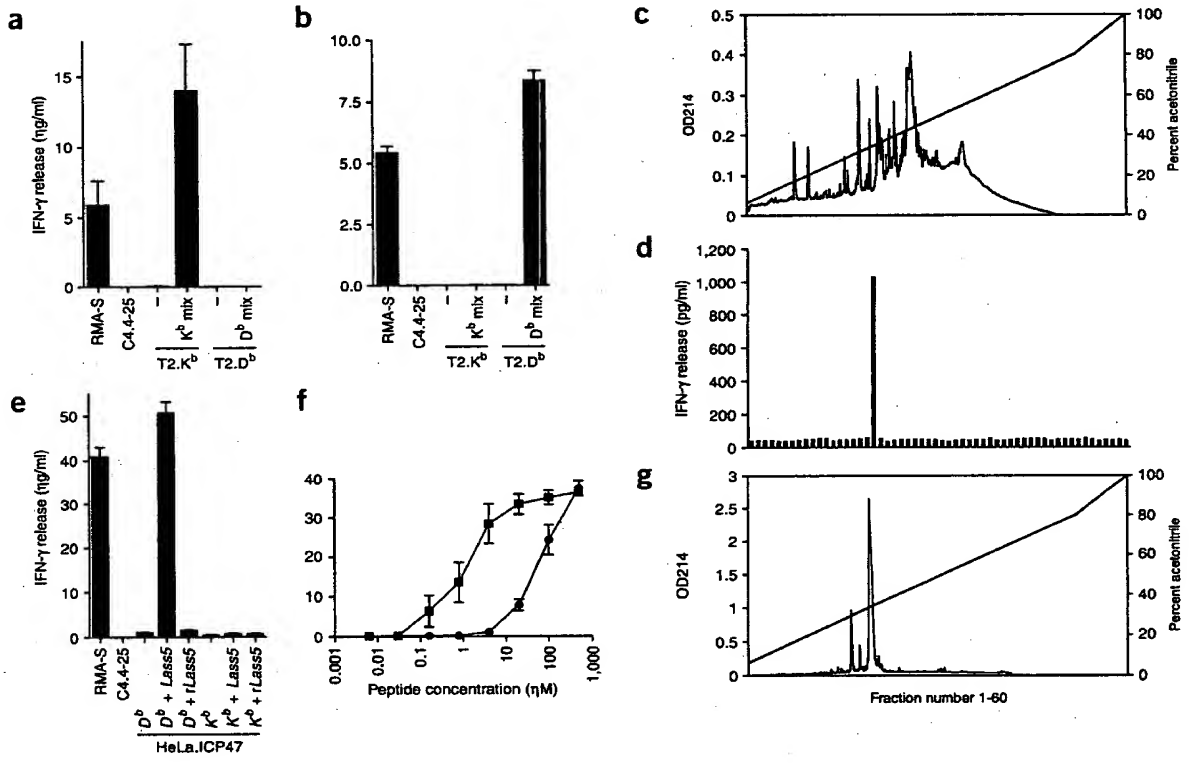

Figure 4 Identification of a D^b-presented TEIPP. (a,b) K^b- (a) and D^b-restricted TEIPP-specific CTLs (b) respond to human TAP-deficient T2 cells with stable expression of K^b or D^b when exogenously loaded with synthetic peptide libraries. Libraries contained peptides with K^b- or D^b-binding motifs[35]. (c,d) D^b molecules from RMA-S cells (10^{11}) were immunoaffinity purified, and acid-eluted peptides were separated by reverse-phase HPLC, applying an increasing acetonitrile gradient (OD214, left axis; percent acetonitrile, right axis) (c). HPLC fractions were loaded onto T2.D^b cells and TEIPP-specific, D^b-restricted CTLs were used to detect the eluted peptide epitope (d). Comparable results were obtained for four separate peptide elutions. Of note, CTL recognition of HPLC fractions depended on the presence of the relevant MHC class I molecule on target cells (data not shown). (e) The MCLRMTAVM peptide is processed and presented from *Lass5*. *Lass5* was cloned from RMA-S cells using gene-specific primers. *Lass5* in the reversed orientation (r*Lass5*) served as control plasmid. HeLa cells expressing the viral TAP inhibitor ICP47 (ref. 37) were transiently transfected with *Lass5* or r*Lass5* in combination with *H-2D^b*– or *H-2K^b*–encoding plasmids. D^b-restricted TEIPP-specific CTLs were used to detect proper processing and MHC class I–restricted presentation of the MCLRMTAVM peptide. Similar results were obtained in three consecutive experiments. (f) Synthetic peptides SLSRLSGTV (circles), retrieved from a bead-assisted peptide library, and MCLRMTAVM (squares) derived from the *Lass5* gene were tested in graded amounts for CTL recognition by the D^b-restricted TEIPP CTLs. (g) HPLC profile of the synthetic MCLRMTAVM peptide using the same acetonitrile gradient as in c. The early peptide peak in the profile contains the MCLRMTAVM sequence of which the C-terminal methionine is oxidized.

Table 1 TCR motif pattern[a]

	Amino acid position							
1	2	3	4	5	6	7	8	9
X^b	A	I	R	L	S	A	T	A
	C	L	K	M	T	G	V	I
	G	M		N		I		L
	I	N				S		M
	L	P				V		V
	M	Q						
	Q	R						
	R	S						
	S	V						
	T	W						
	V							
	Y							
S^c	L	S	R	L	S	G	T	V
M	C	L	R	M	T	A	V	M

[a]Single amino acid substitutions that resulted in equal or increased CTL recognition from Supplementary Table 3 are summarized here. [b]X represents all amino acids. [c]SLSRLSGTV, D^b peptide mimotope MCLRMTAVM, revealed D^b epitope.

MCLRMTAVM peptide is considered as an immunogenic neoantigen by the immune system, although it is derived from a ubiquitously expressed self protein. The same seems to be true for the TEIPPs recognized by the other CTLs isolated so far, because all our CTL cultures selectively react against processing-deficient cells, independent of whether these cells are transformed. The selective presentation on processing-deficient cells of these other peptides may be the result of mechanisms different from the one we proposed for the Lass5-derived epitope, such as suboptimal binding to MHC class I. Irrespective of the underlying mechanism, it is this unique property of TEIPPs that offers the opportunity to exploit these peptides as tumor-specific antigens for the selective, CTL-mediated targeting of processing-deficient tumors *in vivo*.

The peptide sequence MCLRMTAVM is positioned at the C-terminal end of the Lass5 protein, and is part of a domain that protrudes into the lumen of the ER. This may readily explain the proteasome- and TAP-independent processing of this epitope. Generation of TAP-independent CTL epitopes in this manner has been previously described, and has led others to postulate the so-called 'C-end rule'[19,24].

Our results are in line with this rule and, moreover, represent an example of a natural CTL epitope generated through this pathway. A crucial difference between the C-terminal epitope identified by us and those epitopes described previously is its exclusive detection by CTLs at the surface of processing-deficient target cells. Furthermore, our data indicate that TAP-deficient cells display a diversity of peptides derived from self proteins, in that we identified CTL clones with distinct MHC restriction patterns including not only the classical MHC class I molecules K^b and D^b, but also the nonclassical MHC class I molecule Qa-1^b. Although initially this diversity in TEIPP specificities may seem unexpected, it is conceivable that the ER harbors a variety of minor peptide constituents that, because of the presence of more abundant TAP-dependent peptides, are not normally loaded into MHC class I. Examples of CTL restricted by Qa-1^b, or its human homolog HLA-E, have been reported in the context of immune response against alloantigens and pathogen-derived antigens[25]. Our report describes Qa-1^b–restricted CTL responses in the context of antitumor immunity. Although formal proof for the existence of a human TEIPP repertoire is currently lacking, it is conceivable that the ER of human cells also contains MHC class I–binding peptides that

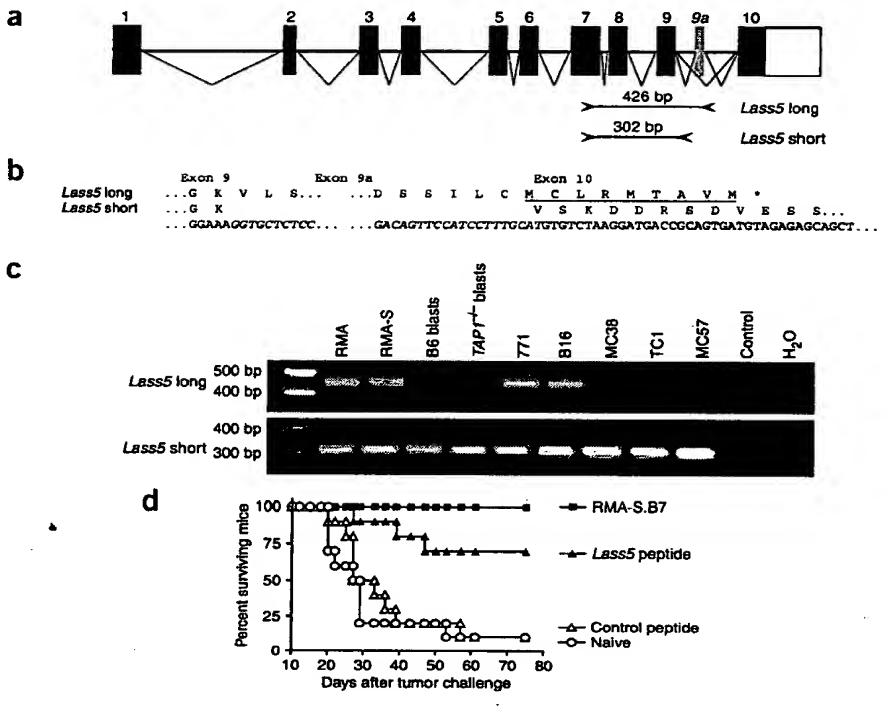

Figure 5 Analysis of *Lass5* expression and vaccination with the *Lass5*-encoded peptide. (a,b) *Lass5* gives rise to two mRNA splice variants discernible upon the inclusion or exclusion of exon 9a (a). Inclusion of exon 9a results in the generation of a long transcript and in a shift in the reading frame of the succeeding exon 10. The TEIPP CTL epitope MCLRMTAVM is therefore exclusively encoded by long transcripts of *Lass5* (b). Notably, the peptide is located at the carboxy terminus of the Lass5 protein because of the presence of an early stop codon in the sequence of the long transcript (b). (c) Short and long transcripts of *Lass5* are detected in all cell lines tested irrespective of their TAP status. Products of RT-PCR using primers as indicated in a were separated on agarose gels. (d) Groups of B6 mice ($n = 10$) were immunized with synthetic peptides comprising the TEIPP D^b peptide MCLRMTAVM or the control D^b peptide SSPVNSLRNVV[38] combined with the T-helper peptide EPLTSLTPRCNTAWNRLKL[21] and CpG oligodeoxynucleotides[20] and challenged with TAP-deficient RMA-S cells. Mice were challenged at day 0 and immunized at days −14, 7 and 14. Immunization with irradiated RMA-S.B7 cells served as a positive control. The experiment was repeated once with comparable results. $n = 10$. The difference between the Lass5 peptide– and control peptide–treated groups is statistically significant (analysis of Kaplan-Meier curves by log rank test, $P = 0.012$).

can reach this compartment independently of proteasome function and TAP function, but that are not normally loaded into MHC class I because of competition by the overwhelming amounts of TAP-dependent peptides. Indeed, human TAP-deficient cells were shown to present a set of TAP-independent peptides in their surface MHC class I molecules[26,27].

Our interest in immune effector cells capable of attacking processing-deficient target cells was initially prompted by the awareness that impairments in the MHC class I processing pathway are frequently observed in human cancers, whereas CTLs are a cornerstone of many anticancer immunotherapies currently under development. Two recent studies have shown that tumors with TAP deficiency are resistant to 'conventional' CTL-mediated attack *in vivo* and, moreover, that these escape variants caused uncontrolled tumor outgrowth in the face of an intact immune system[28,29]. These studies stress the significance of exploring complementary CTL specificities that can eliminate tumor escape variants. Our findings suggest the possibility of developing immunotherapies, such as vaccination strategies, in which activation of the TEIPP-specific CTL repertoire is performed in conjunction with that of the conventional $CD8^+$ CTL and $CD4^+$ T-helper repertoire to prevent immune escape by cancer as a result of processing defects.

METHODS

Cell lines and mice. The origin and culturing of most cell lines used in this study was described previously[30]. C4.4-25$^-$ is a β2m-deficient variant of EL4 (ref. 31). RMA-S.B7-1 is a CD80 transfectant of RMA-S[11]. EC7.1 is a K^b- and D^b-negative variant of RMA-S[15]. We immortalized *Tap1*$^{-/-}$ and wild-type mouse embryo fibroblasts using the adenovirus type 5 E1 gene (clone XC3). We induced *Tap1*$^{-/-}$ and *Tap1*$^{-/-}$*B2m*$^{-/-}$ fibrosarcomas with methylcholantrene (MCB6TAP line). We generated CTL lines and clones as previously described[32]. RMA-specific control CTL clones recognize the TAP-dependent viral peptide CCLCLTVFL ($MuLV^{gag}$) or the tumor peptide NKGENAQAI[30]. For adoptive transfer experiments, we injected B6 mice subcutaneously with 5×10^5 RMA-S tumor cells in 200 μl saline solution. On the same day, we injected 20×10^6 CTLs in 200 μl saline intravenously with 10^5 Cetus Units of recombinant human interleukin (IL)-2 in a subcutaneous depot, emulsified in Incomplete Freund Adjuvant. We administered an additional injection of IL-2 1 week after CTL transfer. For peptide vaccination, we injected mice subcutaneously with 100 μg of peptide, 50 μg T-helper peptide EPLTSLTPRCNTAWNRLKL[21] and 40 μg Toll-like receptor 9 ligand CpG oligonucleotides[20] per mouse in a total volume of 200 μl PBS. We challenged mice subcutaneously in the opposite flank with 8×10^5 RMA-S cells in PBS and they were killed when tumors reached a volume of 1,000 mm^3. All experiments on mice were approved by the ethics committee of the Leiden University Medical Center (DEC, Dierexperimentencommissie).

CTL assays and flow cytometry. CTL activity was measured by chromium (^{51}Cr) release assay or IFN-γ ELISA as previously described[30,32]. Data shown represent mean values obtained from triplicate test wells and error bars represent standard deviation of these values. For antibody blocking, we pretreated CTLs with 20 μg/ml antibodies, washed them and added them as responders to target cells for IFN-γ release. In functional blocking assays, we used antibodies specific for the following: hamster CD3 (Fab_2 fragments of 145-2C11), control hamster TNP-KLH (trinitrophenol–keyhole limpet hemocyanin), rat CD4 (GK1.4) and rat CD8 (2.43). For flow cytometric analysis, we purchased from PharMingen (Becton Dickinson) antibodies specific for the following: CD3 (145-2C11), CD4 (GK1.5), CD8 (Ly2), CD8 (Ly-3.2), Vα2 (B20.1), Vα3.2 (RR5.16), TCR Vβ kit, NK1.1 (PK136), DX5, Thy1 (G7), CD16 (2.4G2), Ly49A (A1), Ly49C/I (5E6), Ly49G2 (LGL-1) and Ly49D (4E5).

Treatment with proteasome inhibitors, IFN-γ or antisense oligonucleotides. To block proteasome function, we incubated targets cells with 20 μM lactacystin, 20 μM NLVS (4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone) or 100 μM LLnL (*N*-acetyl-L-leucyl-L-leucyl-leucyl-L-norleucinal) (Calbiochem), as previously described[32]. We incubated IFN-γ–treated target cells for 48 h with 100 IU/ml of recombinant IFN-γ (Peprotech). We treated MC38 and B16 tumor cells with 'morpholino' oligodeoxynucleotides (ODN)[33] (Gene Tools) comprising the antisense sequence of the 5′-untranslated region of the mouse *Tap1* gene (5′-AGAGTCTGGTCC TAGCCTGGGA-3′). As a control, an ODN comprising the antisense sequence of the 5′-untranslated region of human *TAP1* was used (5′-GGCGAGAAGCT CAGCCATTTAGGG-3′). ODN were loaded into target cells by osmotic shock as recommended by the manufacturer, and assayed after 9 d.

RT-PCR, expression vectors and DNA transfection. For Ly49 RT-PCR, we used the following generic primers: 5′-CAATGGCCCATCTAAACTTG-3′ and 5′-CCAGTTTCTTCCCACAAATACA-3′, generating a product of 149 bp. For *KLRD1* (also known as *CD94*) cDNA, we used the following primers: 5′-ATGGCAGTTTCTAGGATCACTCGG-3′ and 5′-GCTGGAATTCTGCGAA GCACAGA-3′, resulting in a product of 280 bp. The PCR primers for the *KLRC1* (also known as *NKG2*) genes have been published elswhere[34]. The applied PCR primers for cloning full-length *Lass5* long transcript were 5′-ATGGCGACTGCAGCAGCAGCGGAAACCC-3′ and 5′-CTACATCACTGC GGTCATCCTTAGACACATGCAAAGG-3′. We cloned PCR products directly with the TOPO TA cloning kit (Invitrogen) and sequenced them using standard procedures. We detected short and long transcripts of *Lass5* by RT-PCR using the shared upstream primer 5′-GCAGACCCCTTACTGGAAGCTGCC-3′ and the specific downstream primers 5′-CGGTCATCCTTAGACACATGCAAA GG-3′ (long) or 5′-CTGCGGTCATCCTTAGACACCTTTCC-3′ (short). We obtained expression plasmids containing the *Lass5* short and long cDNAs, as well as several other candidate genes (**Supplementary Table 4** and **Supplementary Fig. 3** online) through unidirectional cloning of the sequences concerned into pcDNA3.1 (Invitrogen). We transiently transfected these plasmids into HeLa.ICP47 cells using Lipofectamine 2000 (Invitrogen). We harvested cells 48 h after transfection and used them as stimulator cells for D^b-restricted TEIPP-specific CTLs.

Peptide elution, HPLC, synthetic peptide libraries, mass spectrometry and peptide-binding assay. We eluted peptides out of purified H-$2D^b$ or H-$2K^b$ molecules as previously described[30] with minor modifications. We performed immunoprecipitation with protein A beads covalently coupled with D^b-specific monoclonal antibody 28-14-8S or K^b-specific monoclonal antibody B8-24-3 from lysates of 10^{11} RMA-S cells. We fractionated eluted peptides using reverse-phase micro C2C18 HPLC (Smart System, Amersham). Buffer A was 0.1% trifluoroacetic acid in water; buffer B was 0.1% trifluoroacetic acid in acetonitrile.

We used two distinct types of peptide libraries. We performed the experiments shown in **Figure 4a,b** with soluble peptide libraries based on the K^b- and D^b-binding motifs[35]. The K^b library consisted of peptides with the sequence xxxxFxxI/L/M, whereas the D^b library consisted of peptides with the sequence xxxxNxxxI/L/M (*x* indicates random position for all 20 amino acids). Approximate complexities of these libraries were 10^8 and 10^9, respectively. These peptide mixes were provided by S. Stevanovic (University of Tübingen). The experiments that resulted in the identification of the D^b-binding mimotope peptide (**Fig. 4f**) were performed with a peptide library that was synthesized on hybrid beads. We generated hybrid beads by coupling a mixture of Fmoc-Nle-OH and the 3-(4-hydroxymethylphenoxy)propionic acid ester of Fmoc-Val-OH to a TentagelS amine resin (loading 0.26 mmol/g; particle size, 130 μm). We synthesized a peptide library containing about 650,000 peptides of the structure xLxxxxxV (*x* indicates random position for all 20 amino acids) according to a mix-and-split one bead–one peptide protocol. We divided the peptide library in which each bead contained about 75% acid-labile peptide and 25% acid-stable attached peptide into pools (about 2,200 peptides/well). Of each pool, about one-third of the acid-labile material was cleaved from the beads. After removal of the side chain–protecting groups, we screened the peptide cocktail of each pool for stimulating peptides with TEIPP-specific CTLs using T2.D^b target cells. We subsequently divided hybrid beads from positive pools into new pools in a second (about 24 different peptide sequences/well) and third (1 peptide/well) round of screening. We determined the amino acid sequence of the single stimulating peptide using Edman degradation of the acid-stable portion of the peptide material. We validated the retrieved peptide sequence by resynthesizing the peptide and analysis in CTL assays.

We performed electrospray ionization mass spectrometry on a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF1 and Q-TOF Ultima, Micromass) equipped with an on-line nanoelectrospray source, as previously described[30,36]. We performed tandem mass spectrometry of the Lass5-derived peptide using a Bruker HCT^{plus} Ion-trap. The ion 528.3 *m/z* was selected with a window of 4 Da and sequenced by mass spectrometry–mass spectrometry.

We measured binding of peptides to D^b- and K^b-molecules using the unstable MHC class I molecules expressed at the surface of RMA-S cells that had been preincubated at 26 °C for 48 h, as previously described[28]. The repertoire of instable MHC class I molecules expressed at the RMA-S surface at 26 °C is distinct from that harboring the TEIPP peptides.

URL. EMBL-EBI, http://www.ebi.ac.uk/Databases/protein.html

Accession codes. GenBank: *Lass5*, BC043059.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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